Intracellular Signaling Pathways Required for Rat Vascular Smooth Muscle Cell Migration

Interactions between Basic Fibroblast Growth Factor and Platelet-derived Growth Factor

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Abstract

Intracellular signaling pathways activated by both PDGF and basic fibroblast growth factor (bFGF) have been implicated in the migration of vascular smooth muscle cells (VSMC), a key step in the pathogenesis of many vascular diseases. We demonstrate here that, while bFGF is a weak chemoattractant for VSMCs, it is required for the PDGF-directed migration of VSMCs and the activation of calcium/ calmodulin-dependent protein kinase II (CamKinase II), an intracellular event that we have previously shown to be important in the regulation of VSMC migration. Neutralizing antibodies to bFGF caused a dramatic reduction in the size of the intracellular calcium transient normally seen after PDGF stimulation and inhibited both PDGF-directed VSMC migration and CamKinase II activation. Partially restoring the calcium transient with ionomycin restored migration and CamKinase II activation as did the forced expression of a mutant CamKinase II that had been "locked" in the active state by site-directed mutagenesis. These results suggest that bFGF links PDGF receptor stimulation to changes in intracellular calcium and CamKinase II activation, reinforcing the central role played by CamKinase II in regulating VSMC migration. (*J. Clin. Invest.* 1995. 96:1905–1915.) Key words: calcium/calmodulin-dependent kinase II • cell migration • smooth muscle cells • basic fibroblast growth factor • platelet-derived growth factor

Introduction

Many vascular diseases are characterized by the abnormal accumulation of vascular smooth muscle cells (VSMC)[1] in the tunica intima of blood vessels, a process which is thought to occur in part as a result of the migration of these cells from the tunica media (1, 2). A large number of cytokines, growth factors, and other bioregulatory molecules have been implicated in this phenomena. PDGF is one of the most potent chemoattractants for VSMCs (3). Based on the facts that platelet depletion inhibits intimal thickening in balloon catheter–injured rat arteries without affecting medial VSMC replication (4), that a PDGF-neutralizing antibody causes a 40% reduction in neointimal formation in the same animal model (5), and that marked intimal VSMC accumulation has been observed after exogenous administration of PDGF (6), it is likely that PDGF plays an important role in vivo as a mediator of migration. We previously showed that the migration of VSMCs in vitro is dependent not only on the presence of a gradient of PDGF but also on the phenotypic state of the cells (7). Recently, we demonstrated that the PDGF-directed migration of VSMCs requires activation of the intracellular signaling pathway leading to the activation of calcium/ calmodulin-dependent protein kinase II (CamKinase II) and that modulating the phenotype of VSMCs from the proliferating/synthetic state to one of growth arrest/differentiation inhibits the ability to activate this enzyme in response to PDGF. Activating CamKinase II in growth-arrested VSMCs, by either forced elevation of intracellular calcium or forced expression of a mutant CamKinase II that has been "locked" in the activated state by site-directed mutagenesis, restores the ability of these cells to migrate in response to PDGF (8).

Basic fibroblast growth factor (bFGF), the prototypic member of a family of structurally related growth regulatory polypeptides, is a potent mitogen for a variety of cells (9–12) and has been shown to be an important mediator of VSMC proliferation during the response to balloon-catheter injury in the rat. The administration of neutralizing antibodies to bFGF causes an 80% reduction in medial VSMC proliferation 2 d after injury to the carotid artery (13), while the systemic infusion of recombinant bFGF in rats after balloon catheter denudation increases medial VSMC proliferation rate fivefold (14). It has been proposed that bFGF also plays a critical role in the process of VSMC migration. Treatment of cultured bovine smooth muscle cells with a neutralizing antibody to bFGF reduces their PDGF-stimulated migration by > 50% after in vitro "wounding" of the culture (15), indicating that the chemotactic effect of PDGF requires bFGF. PDGF was also reported to induce a significant increase in bFGF mRNA expression in these cells. In vivo studies (16) support a critical role for bFGF in VSMC migration. The migration of VSMCs from the media to the neointima is dependent on a gradient of PDGF released from platelets attached to the deendothelialized vessel. There is no significant movement of cells after gentle deendothelialization in which the underlying medial layer is not injured unless bFGF is administered immediately after the procedure. Like-

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular free calcium concentration; CamKinase II, calcium/calmodulin-dependent protein kinase II; DMEM, Dulbecco's MEM; D-PBS, Dulbecco's PBS; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IP_3, inositoltriphosphate; PKC, protein kinase C; PLC-γ, phospholipase C-γ; VSMC, vascular smooth muscle cell; WT, wild-type.

wise, the increased migration of VSMCs that accompanies more traumatic injury to the vessel and involves mechanical disruption of the tunica media is prevented by intravenous injection of an antibody that blocks bFGF's biological actions (16).

These observations prompted us to investigate the effects of bFGF on PDGF-induced migration and PDGF-stimulated activation of CamKinase II. We show here that bFGF plays a critical role in the PDGF-directed migration of VSMCs and that it is required for the PDGF-induced activation of CamKinase II. Pretreatment of proliferating VSMCs with a neutralizing antibody to bFGF inhibits the PDGF-directed migration of these cells in a Boyden chamber assay and impairs the ability of PDGF to fully increase intracellular calcium and activate Cam-Kinase II. Cells expressing constitutively activated CamKinase II are insensitive to the effects of bFGF neutralization as are cells treated with ionomycin, a calcium ionophore that elevates intracellular calcium levels and activates CamKinase II independently of either bFGF or PDGF. These results demonstrate that the requirement for bFGF during PDGF-directed VSMC migration is due to the effect of bFGF on PDGF-stimulated release of intracellular calcium and the subsequent activation of Cam-Kinase II. The results reinforce the central role played by Cam-Kinase II activation in regulating VSMC migration.

Methods

Materials. Recombinant human PDGF BB and bFGF (Collaborative Research, Lexington, MA) were dissolved in Dulbecco's modified MEM (DMEM, high glucose; Gibco BRL, Gaithersburg, MD) containing 0.1% BSA and then stored in aliquots at −70°C. Neutralizing antibodies to bFGF were purchased from either Upstate Biotechnology, Inc., Lake Placid, NY, (monoclonal IgG1$_k$) or R and D Systems, Inc., Minneapolis, MN, (polyclonal; IgG fraction) and prepared and stored as suggested by the manufacturers. The two antibody preparations exhibited no significant differences in their ability to block migration, inhibit CamKinase II activation or activity, inhibit bFGF-induced expression of c-*fos* mRNA, or show any cross-reaction and interference with PDGF-induced stimulation of immediate-early gene expression. Ionomycin was obtained from Sigma Chemical Co., St. Louis, MO, and KN-62 from LC Laboratories, Woburn, MA. These reagents were dissolved in DMSO, aliquoted, and stored at −70°C. Calmodulin (Upstate Biotechnology, Inc.) and autocamtide-2 (Peninsula Laboratories, Inc., Belmont, CA) were dissolved in water, aliquoted, and stored at −20°C. All reagents were used only once after thawing.

Cell culture. Medial VSMCs were obtained from the thoracic aortas of 3-mo-old Wistar rats using a modification (7) of the combined collagenase and elastase digestion method (17). The endothelia and adventitia were stripped from the vessel before digestion. Cells were maintained in DMEM/high glucose supplemented with 10% heat-inactivated FBS, 1 mM nonessential amino acids, 20 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 10 μg/ml neomycin (Gibco BRL) in a humidified 5% CO$_2$ atmosphere at 37°C. Proliferating cells were used between the 7th and 14th passages and were harvested for migration assays or biochemical analyses between 50 and 80% confluence. Rat aortic medial VSMC lines containing stably integrated expression plasmids of full-length wild-type (WT) and activated (D3) cDNAs for the rat brain CamKinase II α-subunits have been previously described and characterized (8).

RNA analysis. Total RNA was isolated from VSMC cultures by the guanidinium isothiocyanate procedure (18). Northern blotting analyses were performed as previously described (7) using Church's buffer (19) as both prehybridization and hybridization solutions. The bFGF probe was a cDNA probe encompassing 395 bp of the rat bFGF translated region (20) and was a gift of Dr. William L. Lowe (University of Iowa, Des Moines, IA). The rat glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) probe was a 330-bp cDNA probe obtained by PCR amplification of reverse-transcribed adult rat aorta mRNA using published nucleotide sequences (21). The c-*fos* cDNA probe is also a PCR-generated probe and has been described previously (22). Both c-*fos* and GAPDH probes were completely sequenced by the dideoxy-mediated chain termination method (23) to verify complete matching with published DNA sequences. Northern blots were analyzed on a blot analyzer (Betascope 603; Betagen Corp., Waltham, MA) for quantitative analysis.

bFGF immunoassay. VSMCs were cultured in 35-mm culture dishes, treated with 10 ng/ml PDGF BB for the times indicated, and then harvested (24) by first rinsing the cultures in Dulbecco's PBS (D-PBS) and then overlaying them with 0.2 ml of D-PBS containing 2 mM EDTA, pH 8.0, 2 mM *N*-ethylmaleimide, and 1 mM PMSF. The plates were then subjected to four freeze/thaw cycles (liquid nitrogen, 10 min; 37°C, 15 min). Extracts were collected into microcentrifuge tubes (1.5 ml) and clarified by centrifugation at 14,000 rpm for 15 min at 4°C. Protein content of the extracts was assessed using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). The cell lysates were stored at −80°C until bFGF quantification was performed using a commercially available solid phase ELISA (Quantikine FGF Basic Immunoassay Kit; R and D Systems).

VSMC migration assay. Migration assays were performed using a modified Boyden chamber as previously described (7, 25). Polycarbonate filters (13-mm diameter, 8-μm pores, Nuclepore Corp., Pleasanton, CA) were first coated overnight with 100 μg/ml type I collagen (Upstate Biotechnology Inc.). Growth factors, chemoattractants, and other reagents were used at the concentrations indicated in the results and added when the cells were added to the upper chamber of the Boyden apparatus. To distinguish between chemotactic and chemokinetic effects, migration was compared under situations in which the chemoattractant was added only to the lower chamber of the Boyden chamber (chemotaxis) or to both upper and lower chambers (chemokinesis). The effects of the various cell treatments on cell attachment to the upper side of the filter were routinely monitored by counting cells on the filters after fixation and staining (7). For these attachment studies, only 50,000 cells (instead of the 200,000 cells routinely added for a migration assay) were plated onto the filter.

Affinity immunoabsorbtion of antibody preparations. Mouse monoclonal bFGF-neutralizing antibody, bFM-1 (26) (05-117; Upstate Biotechnology Inc.), or rabbit polyclonal neutralizing antibody (R and D Systems) were resuspended in buffer H (10 mM Tris, pH 7.5, 500 mM NaCl, and 1 mM PMSF) and 100 μg incubated with 500 μl of a 1:1 suspension of heparin-Sepharose (Sigma Chemical Co.) that had been preincubated with either 1% BSA alone or 1% BSA and 5 μg of recombinant human bFGF (Collaborative Research). The antibodies were allowed to incubate with the bFGF- or BSA-charged heparin-Sepharose for 2 h at room temperature, at which time the supernatant was collected by centrifugation and reexposed for two more hours to a second batch of bFGF- or BSA-charged heparin-Sepharose. The supernatant was then collected and incubated for 1 h at room temperature with 50 μl of a 50% suspension of protein A and protein G conjugated to agarose (20 mg IgG/ml binding capacity of packed beads; Oncogene Science, Inc., Mineola, NY). Antibodies were eluted with 100 μl of 100 mM glycine and then immediately neutralized with 10 μl 1 M Tris, pH 8. Antibody preparations that had not been exposed to bFGF- or BSA-charged heparin-Sepharose, but were precipitated by protein A/G agarose, served as a positive control for the procedure. Total antibody content of the protein A/G agarose eluants was estimated from serial dilutions blotted onto polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA) and detected with horseradish peroxidase–conjugated anti–mouse or anti–rabbit Ig.

CamKinase II activation and activity assays. Activation of CamKinase II in response to PDGF and other reagents was measured as autophosphorylation. This was assessed by following the incorporation of radioactive phosphate into CamKinase II as described previously (8). VSMC cultures were labeled for 3–5 h with 250 μC/ml of ^{32}P-labeled sodium phosphate, pH 7.2, and then prepared for immunoprecipitation

with a specific antibody to the δ isoforms of CamKinase II (generously provided by Dr. Harold Singer, Weis Center for Research, Danville, PA). Previous studies have established that δ-CamKinase II isoforms account for most, if not all, of the CamKinase II in rat VSMCs (27). CamKinase II activity in cellular extracts was measured using a synthetic peptide substrate (Autocamtide 2; Peninsula Laboratories [8]). It is expressed as the percent autonomous activity and is measured as the percent activity that is calcium/calmodulin independent relative to the activity in the presence of excess calcium and calmodulin (i.e., total activity).

Intracellular calcium measurements. VSMCs were cultured on glass coverslips and incubated in 25 μM of the acetoxymethyl ester derivative of indo-1 (Molecular Probes, Inc., Eugene, OR) for 45 min at 37°C. Ca^{2+}-dependent indo-1 fluorescence was measured in a small number (10–20) of indo-1–loaded cells bathed in 20 mM Hepes, pH 7.4, 15 mM glucose, 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, and 1.2 mM $MgSO_4$ at 25°C. Fluorescence was excited by epiillumination with 10-μs flashes of 350±5-nm light. Indo-1 emission, in spectral windows of 390–434 and 477–507 nm (corresponding to the Ca^{2+}-bound and Ca^{2+}-free forms of the indicator, respectively) were simultaneously collected via paired photomultipliers (28). The ratio of these two channels was used to estimate intracellular calcium concentration ($[Ca^{2+}]_i$) (29).

Confocal microscopy. VSMCs were cultured on glass coverslips and treated with 10 ng/ml PDGF BB for 8 h. The cells were then fixed overnight at 4°C in 3.7% formaldehyde in D-PBS. After washing with D-PBS containing 1% BSA (DPBS-BSA), the cells were exposed for 1 h to 10 μg/ml of a mouse mAb to bFGF (05-118; Upstate Biotechnology, Inc.,). The coverslips were washed in DPBS-BSA for 30 min and then incubated in the dark for 1 h with 10 μg/ml of an anti–mouse IgG antibody conjugated to FITC (Sigma Chemical Co.). Coverslips were mounted on slides for confocal microscopy. The instrument (MRC 600; Bio-Rad Laboratories, Richmond, CA) was equipped with a laser head (Ion Technology, Inc., Fort Collins, Co) and used a computerized stage for z-axis movements. The excitation and emission wavelengths used were 488 and 530 nm, respectively. Confocal images focused on 1-μm-thick planes at different levels along the axis parallel to the plane of the slide.

Statistical evaluations and comparisons. All data are expressed as the mean±SE. Student's unpaired *t* test was used to compare the data presented in Fig. 8. The comparison of the mean values of the control and treated groups for the remaining figures was made using the ANOVA with *P* values corrected by the Bonferonni method (30). For these measurements, $P < 0.01$ was considered statistically significant.

Results

PDGF increases bFGF mRNA and bFGF synthesis/release from VSMCs. While others have described increased release of bFGF from VSMCs 24–48 h after stimulation with PDGF (31), we measured PDGF-induced changes in bFGF mRNA levels and protein production over a time course compatible with migration assays in the Boyden apparatus (4 h). Fig. 1 *A* shows that unstimulated VSMCs did not express detectable levels of bFGF mRNA as measured by Northern blotting analysis. mRNA for bFGF, however, was observed after 3 h of PDGF treatment. bFGF mRNA levels remained visible after 8 h of PDGF BB incubation but returned to untreated levels by 24 h. bFGF protein levels also increased after PDGF stimulation (Fig. 1 *B*). At 4 h, protein levels were approximately twofold greater than unstimulated controls. Protein levels continued to increase, peaking approximately fourfold above unstimulated levels (27.6±1.7 vs. 7.4±0.3 pg/μg protein) at 8 h and continuing to remain elevated for at least 24 h.

Neutralizing antibodies to bFGF block PDGF-directed migration. To evaluate the effect of bFGF on PDGF-directed mi-
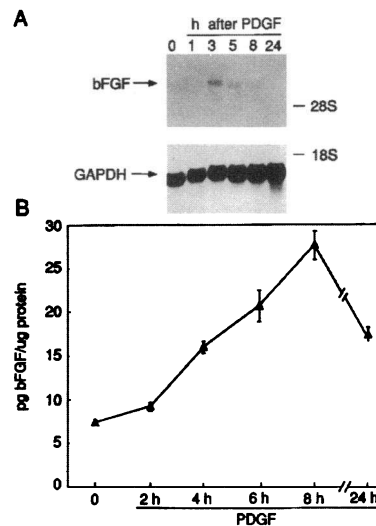

Figure 1. PDGF stimulates bFGF mRNA and protein. (*A*) Northern blotting analysis of changes in bFGF and GAPDH mRNA after incubation of VSMCs with PDGF BB. Total RNA was isolated from VSMCs treated with 10 ng/ml PDGF BB at the times indicated. 10 μg of RNA was then subjected to denaturing electrophoresis and prepared for hybridization with a ^{32}P-labeled cDNA probe to rat bFGF as described in Methods. The blot was then stripped in 5% SDS and reexposed to a ^{32}P-labeled cDNA probe to rat GAPDH. The bFGF blot was exposed to x-ray film for 24 h, while the blot probed with GAPDH was exposed for only 8 h. (*B*) Immunoassay of rat bFGF in extracts of the cell and extracellular matrix from PDGF BB–stimulated VSMCs. Extracts were prepared from cultures treated with 10 ng/ml PDGF BB and assayed for immunoreactive bFGF and total proteins. Results from four to five independent experiments are presented as the mean±SE. No immunoreactive bFGF was detected in conditioned media from the cultures even after enrichment of the media by heparin agarose chromatography (24).

gration, VSMCs were treated during the course of the Boyden chemotaxis assay with antibodies that neutralize bFGF. The results of these experiments are shown in Fig. 2. The data in bar 2 show that significantly more cells migrated to the underside of the filter with PDGF in the lower chamber of the Boyden apparatus than with BSA alone (bar *1*). The addition of 1 μg/ml of neutralizing mAb to the upper chamber (bar *3*) reduced the number of cells that migrated toward the PDGF by ~ 58% (70.7±4.7 vs. 29.6±3.7 cells/field; $P < 0.001$). (These and all subsequent comparisons were made after subtraction of BSA background, which was 9.6±0.7 cells/field in the series of experiments in Fig. 2). When 5 μg/ml of antibody was added (bar *4*), the effect was even greater (83% inhibition; 70.7±4.7 vs. 12.7±5.3 cells/field; $P < 0.001$). Similar results were obtained with a polyclonal neutralizing antibody (R&D Systems) (bars *6* and *7*). No significant effect on migration was observed when either mouse Ig of the same isotype as the monoclonal bFGF antibody (bar *5*) or the IgG fraction from nonimmune rabbit serum (bar *8*) was added to the cells. Neither of the antibodies affected the attachment of VSMCs to the filter separating the upper and lower chambers of the Boyden apparatus. When expressed relative to cell attachment in untreated wells, 99±5 and 104±2% of the cells treated with the monoclonal neutralizing antibody were attached 1 h and 4 h, respectively, after plating in the chamber.

To exclude any cross-reaction between the bFGF neutralizing antibodies and PDGF, we examined their effect on the ability of PDGF to induce c-*fos* mRNA. Fig. 3 shows a representative Northern blot of this analysis. After 30 min of incubation with 10 ng/ml PDGF BB, c-*fos* mRNA levels increased dramatically (compare lanes *1* and *2*). Preincubation with a PDGF-neutralizing antibody reduced the c-*fos* response to PDGF BB
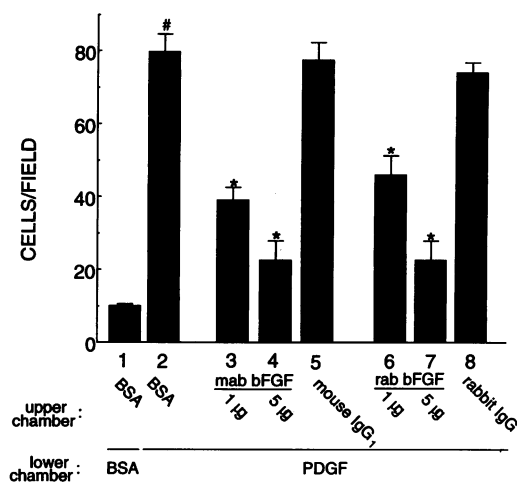
Figure 2. Neutralization of endogenous bFGF inhibits PDGF-directed VSMC migration. Migration was measured in proliferating VSMCs as chemotaxis toward 10 ng/ml PDGF BB. The migration of cells is from the upper to lower chamber of the apparatus. Additions to these chambers are indicated below the data bars. Results are expressed as the number of cells per high power (×400) field. (Bar *1*) BSA in both the upper and lower chambers. (Bar *2*) PDGF as chemoattractant in the lower chamber (PDGF is present in the lower chamber for all other data points in the figure). (Bars *3* and *4*) addition of either 1 µg/ml (bar *3*) or 5 µg/ml (bar *4*) neutralizing mouse mAb (*mab*) to bFGF. (Bar *5*) isotype-matched control for the mouse monoclonal neutralizing antibody at 5 µg/ml. (Bars *6* and *7*) addition of either 1 µg/ml (bar *6*) or 5 µg/ml (bar *7*) neutralizing rabbit polyclonal antibody (*rab*) to bFGF. (Bar *8*) 5 µg/ml IgG fraction from rabbit nonimmune serum. Data are expressed as mean±SE of at least five different experiments. Statistical comparisons: $^{\#}P < 0.001$ compared to BSA (bar *1*); *P < 0.001 compared to migration of VSMCs toward PDGF with no pretreatment (bar *2*).

Figure 4. Inhibition of PDGF-directed VSMC migration by affinity-purified bFGF antibodies. Migration was measured in proliferating VSMCs as chemotaxis toward 10 ng/ml PDGF BB. Results are expressed as the percent inhibition of migration seen with unfractionated neutralizing bFGF antibodies. (Bars *1–4*) data for the mouse monoclonal neutralizing bFGF antibody; (Bars *5–8*) data for the rabbit polyclonal neutralizing bFGF antibody, (Bars *1* and *4*) inhibition after protein A/protein G affinity purification. (Bars *2* and *6*) inhibition after absorption of antibody preparations against bFGF immobilized on heparin-Sepharose followed by protein A/G affinity purification. (Bars *3* and *7*) inhibition after absorption of antibody preparations against heparin-Sepharose followed by protein A/G affinity purification. (Bars *4* and *8*) inhibition with 5 µg/ml unfractionated antibody applied to both upper and lower chambers. Antibody concentration was estimated by dot-blot analysis as described in Methods. Data are expressed as the mean±SE of three different experiments. $^{\#}P > 0.01$ (NS) compared to unfractionated neutralizing bFGF antibodies (arbitrarily taken as 100%).

by $> 50\%$ (lane *4*), while bFGF antibodies had no significant effect on this induction (lane *3*). On the other hand, addition of the bFGF antibody completely blocked the increase in c-*fos* mRNA levels seen after stimulation by exogenous bFGF (compare lanes *5* and *6*). Therefore, the bFGF antibodies did not interfere with the ability of the VSMCs to respond to the chemoattractant, PDGF BB.

To exclude the possibility that the antibodies were reacting with either unknown growth factors or with substances that were actually responsible for the effects observed, both antibody
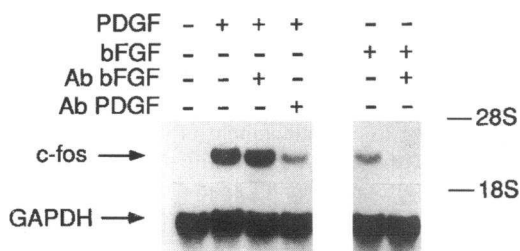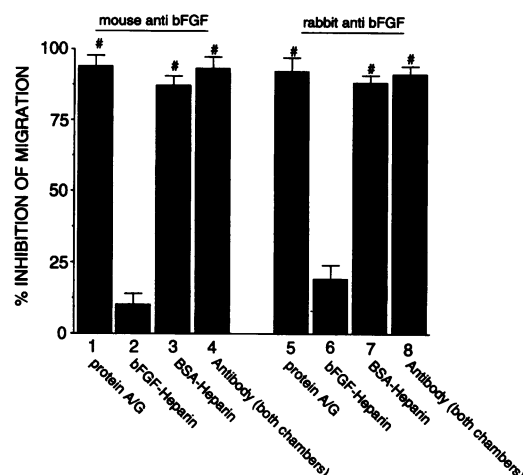

Figure 3. Neutralizing antibodies to bFGF do not interfere with PDGF-induced early gene expression. Total RNA was isolated from VSMCs 30 min after incubation with either PDGF BB (10 ng/ml) or bFGF (10 ng/ml) alone or in combination with the indicated antibodies. Neutralizing antibodies (5 µg/ml) to either PDGF or bFGF were applied 30 min before the addition of PDGF and bFGF to the cultures.

preparations were preabsorbed with immobilized recombinant bFGF before their addition in the migration assay. The results of these experiments are shown in Fig. 4, in which the ability of the various affinity-purified fractions are compared with unfractionated antibody. For both antibodies, affinity purification over a mixture of protein A and G (bars *1* and *5*) did not significantly affect the ability of the antibodies to inhibit migration. Incubation of the antibodies with bFGF-charged heparin-Sepharose followed by protein A/G purification, however, substantially reduced the inhibitory effects of both antibodies (bars *2* and *6*). As a control, both antibodies were also incubated with heparin-Sepharose incubated with BSA alone and then purified by protein A/G. These preparations were comparable in their ability to inhibit migration to that of untreated antibodies (bars *3* and *7*), demonstrating that the effect of affinity purification is due primarily to absorption of antibodies reacting with bFGF and not with the heparin used to immobilize bFGF.

Although the data shown in Fig. 4 (bars *1* and *5*) exclude the possibility that VSMC chemoattractants present in the antibody preparations were short-circuiting the PDGF gradient, antibodies were added to both sides of the chamber in a series of experiments. The effect of adding antibody to both sides of the chamber was not significantly different from the inhibitory effect seen with antibody added only to the top chamber (bars *4* and *8*).

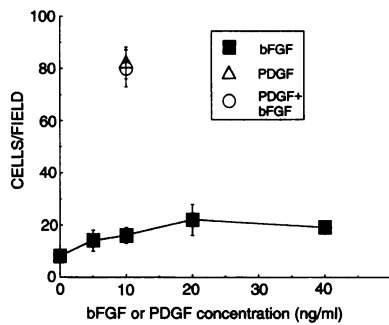When placed in the bottom chamber of the Boyden appara-

Figure 5. bFGF dose response as a chemoattractant. Migration of VSMCs toward increasing concentrations of bFGF expressed as the number of cells per high power field (*filled squares*). For comparison, the migration of VSMCs in response to 10 ng/ml PDGF BB is shown (*open triangle*). The presence of bFGF in the lower chamber (*open circle*) had no effect on PDGF-directed migration. Data are expressed as mean±SE.

tus, bFGF was a weak chemoattractant even over a broad range of concentrations (Fig. 5, *closed squares*). While 10 ng/ml PDGF BB stimulated migration seven to eight times over that seen with BSA alone (*open triangle*), bFGF-directed migration at 20 ng/ml stimulated migration was only 1.8 times that of the BSA control. When added together with PDGF BB in the bottom chamber (*open circle*), bFGF had no additional effect on migration.

Neutralizing antibodies to bFGF block CamKinase II activation and activity. We have previously demonstrated that the activation of CamKinase II is a requirement for the PDGF-directed migration of VSMCs (8). The effects of the neutralizing antibodies described above on cell migration led us to examine whether endogenous bFGF was involved in CamKinase II activation and/or activity. Activation of CamKinase II occurs as a result of autophosphorylation and was monitored by following the incorporation of radiolabeled phosphate into the enzyme. Cells were incubated with radiolabeled inorganic phosphate, treated with various growth factors and reagents, and a cellular extract was prepared. CamKinase II was immunoprecipitated from the extract and identified by autoradiography after separation of the immunoprecipitates by SDS-PAGE. The amount of radiolabeled phosphate in the CamKinase II band was then quantified. Fig. 6 *A* and *B*, shows representative autoradiographs of the cellular extracts after SDS-PAGE separation. As shown, the incorporation of radiolabeled phosphate into cellular CamKinase II dramatically increased with either a 1-min incubation with PDGF BB or a 2-min incubation with ionomycin. Incubation with bFGF alone, however, did not lead to labeling of CamKinase II. Preincubation of the cells with the neutralizing monoclonal bFGF antibody at a dose sufficient to block migration (5 μg/ml) almost completely blocked CamKinase II phosphorylation in response to PDGF. A graphical representation of the results of this experiment and others is shown in Fig. 6 *C* (*open bars*). For comparison, the basal level of activation was arbitrarily assigned a value of 1. PDGF and ionomycin increased the incorporation of phosphate into the enzyme by 4.5±0.3- and 3.4±0.5-fold, respectively ($P < 0.001$). Preincubation with bFGF neutralizing antibodies for 30 min significantly decreased CamKinase II phosphorylation in response to PDGF (1.7±0.2 vs 4.5±0.3; PDGF/antibody vs PDGF alone; $P < 0.001$) to a level not significantly different from untreated control ($P > 0.01$) or incubations with the antibody alone (1.2±0.4; $P > 0.01$). Likewise, the level of CamKinase II phosphorylation in response to bFGF was not significantly different from that of untreated samples.
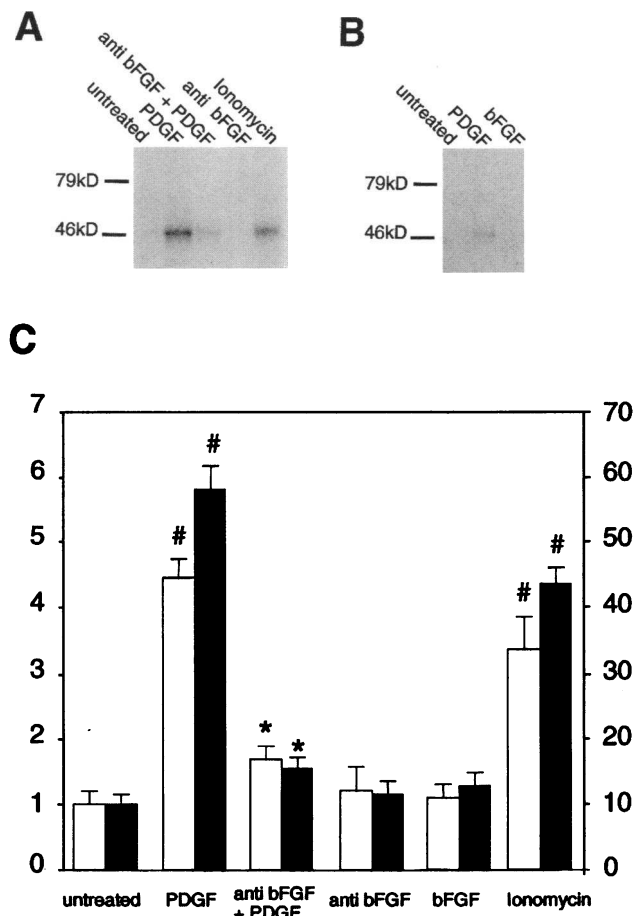

Figure 6. Activation and activity of CamKinase II in VSMCs treated with neutralizing antibodies to bFGF. (*A*) and (*B*) Autoradiograms of ^{32}P incorporation into CamKinase II in proliferating VSMCs treated with 10 ng/ml PDGF BB, PDGF BB and 5 μg/ml antibody to bFGF (applied 30 min before the addition of PDGF), anti-bFGF alone, 1 μM ionomycin, or 10 ng/ml bFGF alone. Except for ionomycin, extracts were prepared for immunoprecipitation with a δ-CamKinase II–specific antibody 1 min after growth factor addition. Ionomycin-treated cells were extracted 2 min after incubation. The positions of the 79-kD and 46-kD prestained markers are indicated to the left. (*C*) Bar graph of ^{32}P incorporation into CamKinase II (*open bars*) and the percent autonomous CamKinase II activity (*filled bars*) in untreated cells, and cells treated with 10 ng/ml PDGF BB, PDGF BB (10 ng/ml) and antibody to bFGF (5 μg/ml), antibody to bFGF alone (5 μg/ml), bFGF (10 ng/ml), and ionomycin 1 μM. Results are expressed relative to untreated (*control*) levels of ^{32}P incorporation (left *y*-axis) or CamKinase II calcium-independent (or autonomous) activity (right *y*-axis). Data are expressed as mean±SE of at least three to four different experiments. Statistical comparisons: #$P < 0.001$ vs. untreated cells, *$P < 0.001$ vs. PDGF-stimulated cells.

Similar results were obtained when CamKinase II enzyme activity, expressed as percent autonomous (i.e., calcium-independent) activity, was measured using a synthetic substrate (Fig. 6 *C; filled bars*). Under unstimulated, basal conditions, 9.9±1.6% of total CamKinase II activity was found to be calcium independent. After the addition of PDGF or ionomycin, calcium-independent activity increased significantly (58.8±3.6% for PDGF and 44±2.5% for ionomycin). When the cells were preincubated for 30 min with bFGF-neutralizing antibodies, PDGF was no longer able to significantly increase
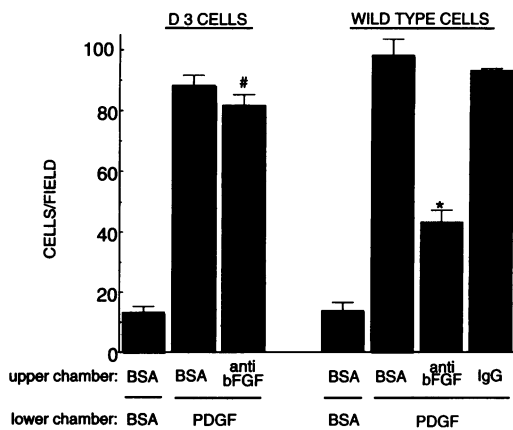
Figure 7. VSMCs expressing constitutively activated CamKinase II are unaffected by bFGF neutralizing antibodies. Migration expressed as number of cells/field (×400). D3 cells are VSMC stable transfectants expressing constitutively activated α-isoform of CamKinase II, while WT cells are stable transfectants expressing unmodified α-CamKinase II (8). The bFGF neutralizing monoclonal antibody or matched IgG isotype controls were added at 5 µg/ml to the upper chamber of the Boyden apparatus 30 min before the addition of PDGF to the lower chamber. Data are expressed as mean±SE of at least five different experiments. Statistical comparisons: # no significant difference (P > 0.01) compared to untreated, PDGF BB–stimulated D3 cells, *P < 0.001 vs. untreated, PDGF BB–stimulated WT cells.

the activity of the enzyme above basal levels (15.6±1.8%, P > 0.01 compared to control). The data in Fig. 6 C also show that bFGF on its own was not able to stimulate an increase in CamKinase II activity (12.8±2.2%; P > 0.01). Together, these results demonstrate that bFGF-neutralizing antibodies that block migration also block CamKinase II activation and activity in response to the chemoattractant, PDGF, an event previously shown to be essential for VSMC chemotaxis (8).

VSMCs expressing constitutively activated CamKinase II are resistant to the effects of bFGF neutralization. If the neutralizing antibodies reduce PDGF-directed VSMC migration due to their ability to block the activation of CamKinase II, which normally occurs upon the addition of PDGF, then the migration of VSMCs overexpressing constitutively active (i.e., calcium/calmodulin-independent) CamKinase II cDNA should be unaffected by the antibodies. Fig. 7 shows the effect of bFGF neutralizing antibodies on the migration of VSMC cell lines containing stably integrated cDNAs for either WT or mutant (D3) CamKinase II α-subunits from rat brain. The mutation involved replacement of amino acids T286 and V287 with two aspartic acid (D) residues. T286 is the site of the autophosphorylation reaction involved in the activation of CamKinase II to its calcium/calmodulin-independent state and the amino acid substitutions described above introduce two constitutively negative charges into this region, mimicking the effect of autophosphorylation and producing a permanently activated CamKinase II molecule (8, 32). The migration of these two stable cell lines toward PDGF was slightly greater than that of untransfected or vector alone–transfected cells (Fig. 7; WT: 98.3±5.5 cells/field, D3: 88.3±3.3 cells/field, and untransfected control: 79.7±4.7 cells/field). Although WT cells expressed significantly more CamKinase II than untransfected cells, this additional enzyme is not activated in the absence of PDGF (8). Neutralizing mAb to bFGF still inhibited PDGF-directed migra-
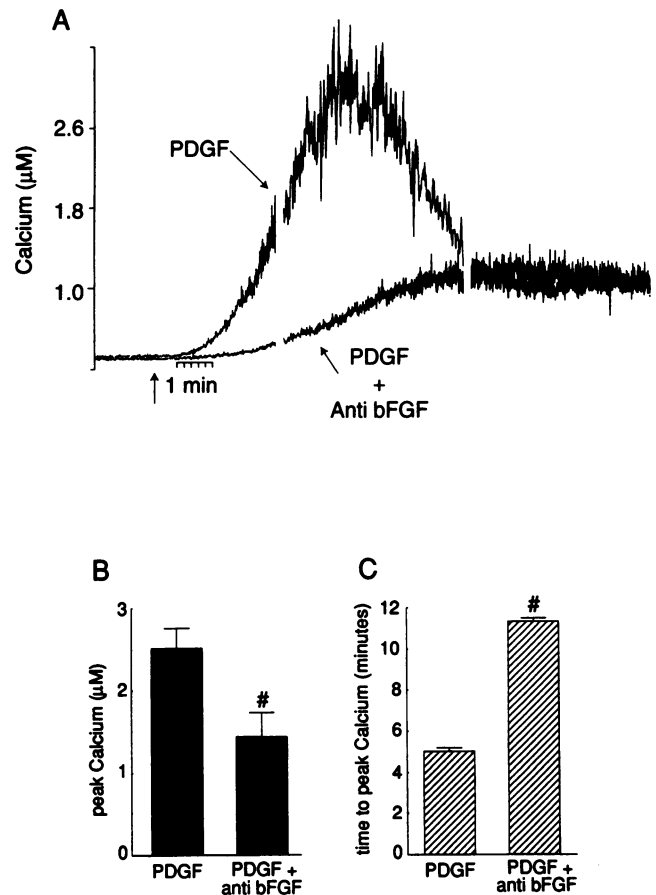

Figure 8. Changes in [Ca^{2+}]$_i$ in PDGF-stimulated VSMCs pretreated with bFGF neutralizing antibodies. (*A*) [Ca^{2+}]$_i$ responses to PDGF in PDGF-stimulated VSMCs pretreated for 30 min with 5-µg/ml monoclonal neutralizing bFGF antibody (*PDGF + anti bFGF*) or isotype-matched control (*PDGF*) as estimated from changes in intracellular indo-1 fluorescence. (*B*) and (*C*) Peak [Ca^{2+}]$_i$ (*B*) and time to peak [Ca^{2+}]$_i$ (*C*) in PDGF-stimulated VSMCs pretreated with 5 µg/ml bFGF monoclonal neutralizing antibody (*PDGF + anti bFGF*) or isotype matched control (*PDGF*). Data are expressed as mean±SE. Statistical comparisons: #P < 0.001 vs. PDGF + isotype control.

tion (64% inhibition; 43±4.4 cells/field, P < 0.001) in these cells, while isotype-matched control Ig (for the mAb) had no effect on the migration of WT cells. As predicted, the monoclonal neutralizing antibody was completely ineffective in the mutant D3 cells overexpressing constitutively activated CamKinase II (Fig. 7; 93.3±0.7 cells/field; P > 0.2, NS from PDGF-stimulated cells not treated with antibody).

Neutralizing antibodies against bFGF interfere with the intracellular calcium response to PDGF. PDGF BB causes a rapid and large increase in intracellular free calcium concentration ([Ca^{2+}]$_i$ in proliferating VSMCs (8). Both the activation and increased activity of CamKinase II in response to chemoattractants such as PDGF BB are dependent on the presence of intracellular calmodulin and increased [Ca^{2+}]$_i$. To determine whether the neutralizing bFGF antibodies affected the [Ca^{2+}]$_i$ responsiveness to PDGF, we measured changes in indo-1 fluorescence in VSMCs in response to PDGF in the presence or absence of the antibodies. Fig. 8 A shows the changes in [Ca^{2+}]$_i$ that occur in VSMCs after adding PDGF. These changes are
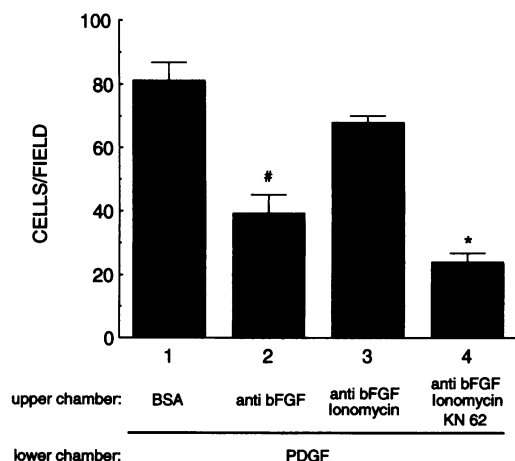
Figure 9. Ionomycin restores migration in VSMCs blocked by bFGF-neutralizing antibodies through a CamKinase II–dependent mechanism. Migration with 10 ng/ml PDGF BB used as chemoattractant in the lower chamber. Antibody to bFGF was added to cells 30 min before the addition of VSMCs to the upper chamber and ionomycin (1 μM) and KN-62 (10 nM) were added to the upper chamber at the time of cell addition. Ionomycin was removed after 20 min, while KN-62 remained in the chamber throughout the migration experiment. Data are expressed as mean±SE of at least five different experiments. Statistical comparisons: *#*$P < 0.001$ compared to untreated, PDGF BB–stimulated cells (bar *1*), *$P < 0.001$ vs. PDGF-stimulated VSMCs treated with antibody to bFGF and ionomycin (bar *3*).

conveniently described as having two components: a transient increase in $[Ca^{2+}]_i$ that rapidly reaches a peak followed by a sustained or tonic elevation at a level significantly lower than the peak response. Preincubation with bFGF-neutralizing antibodies before PDGF addition significantly blunted the initial rapid rise in $[Ca^{2+}]_i$ but did not affect the tonic component of the response. Fig. 8 *B* shows the differences in peak calcium levels attained under these two conditions and Fig. 8 *C* the time to reach that peak. Treatment with PDGF alone resulted in a peak calcium of 2.51±0.25 μM that was achieved rapidly (5±0.2 min), while preincubation with the antibody decreased the peak calcium level to 1.43±0.3 μM ($P < 0.001$) and this level was achieved only after 11.3±0.2 min ($P < 0.001$).

The $[Ca^{2+}]_i$ determinations described above suggested that changes in intracellular calcium dynamics associated with preincubation of bFGF antibodies were responsible for the failure of antibody-treated cells to activate CamKinase II in response to PDGF. Incubating the cells with a calcium ionophore to elevate $[Ca^{2+}]_i$ should then bypass the effects of the bFGF-neutralizing antibodies. These experiments are shown in Fig. 9. Addition of ionomycin (bar *3*) overrode much of the inhibition in migration after antibody pretreatment (bar *2*) (39±6.1 and 67.7±2.3 cells/field for antibody alone and ionomycin/antibody, respectively; $P < 0.001$) and returned migration to a level not significantly different from untreated cells (bar *1*) (81±5.9 cells/field, $P > 0.05$). This effect of ionomycin was probably due to its ability to activate CamKinase II by raising $[Ca^{2+}]_i$ because the addition of KN62, a specific inhibitor of CamKinase II activation (8, 33), blocked the ability of ionomycin to override the effects of bFGF antibody pretreatment (bar *4*) (23.8±3.0 cells/field).

bFGF is present in the extracellular matrix surrounding VSMCs. The above results suggest that bFGF is important for

PDGF-directed VSMC migration and that endogenously produced bFGF is accessible to the neutralizing antibodies used in this study. While the data in Fig. 1 demonstrate that bFGF is produced by VSMCs, especially in response to PDGF, they do not indicate whether the growth factor is actually secreted by these cells, an important consideration given the fact that bFGF does not contain a secretory signal sequence (34). Attempts to identify bFGF in the conditioned media of PDGF-treated VSMCs using an enzyme-linked immunoassay with a sensitivity greater than most bioassays (5 pg/ml ELISA vs 10–20 pg/ml for NR6-3T3 proliferation assay [35]) were all negative, suggesting that newly produced bFGF was either confined to the cells and/or secreted and bound to the extracellular matrix. To localize bFGF, confocal microscopic imaging of proliferating VSMCs with bFGF antibodies was performed (Fig. 10). Images of the cells were taken at the plane of the culture dish (level 0 μm) and at a level through the cell and its nucleus (level 2 μm) 8 h after treatment with PDGF BB. Fig. 10, *C* and *E* shows staining at these levels with the bFGF antibody, while Fig. 10 *B* and *D*, shows negative controls with isotype-matched Ig. A comparison of Fig. 10 *E* and *G* (a phase contrast image) indicates that bFGF at this level is present predominantly in the area immediately outside the cell. Fig. 10 *C* indicates that bFGF is also present in the cytosol and at a much lower level in the cell nucleus. These results demonstrate that bFGF is present in both the cytosol and extracellular matrix surrounding VSMCs.

Discussion

The current studies were undertaken to elucidate the role that bFGF plays in PDGF-directed VSMC migration and to identify the intracellular signaling events involved in this process. Our data demonstrate that the requirement for bFGF is due to its effects on the ability of PDGF to activate CamKinase II. We recently reported that the migration of VSMCs toward PDGF BB is mediated by and regulated through activation of CamKinase II (8). Those studies showed that migrating VSMCs activate CamKinase II in response to PDGF BB, that inhibiting CamKinase II activation with a specific reagent (KN-62) blocks the migration of these VSMCs, and that growth-arrested VSMCs which do not migrate toward PDGF BB do not activate CamKinase II. Forced activation of CamKinase II in such growth-arrested VSMCs, achieved either by elevating intracellular calcium with an ionophore or by transfection of a constitutively activated CamKinase II cDNA, restores migration. In the current study, we show that migration of VSMCs toward PDGF is inhibited by antibodies that neutralize extracellular bFGF and subsequently prevent CamKinase II activation by PDGF. This conclusion is supported by the following observations: (a) neutralizing bFGF antibodies not only blocked PDGF-directed VSMC migration but also PDGF-induced CamKinase II activation and enzyme activity; (b) ionomycin, a calcium ionophore which causes an abrupt increase in intracellular calcium levels, overrode the effects of bFGF neutralization on PDGF-directed cell migration, and this effect of ionomycin was blocked by KN-62; and (c) mutant VSMCs that express constitutively active CamKinase II were resistant to the effects of bFGF neutralization.

The effects of bFGF on cell proliferation are widely recognized (36–41). Mitogenic stimulation by bFGF has also been described for VSMCs (42, 43), and a key role of bFGF in
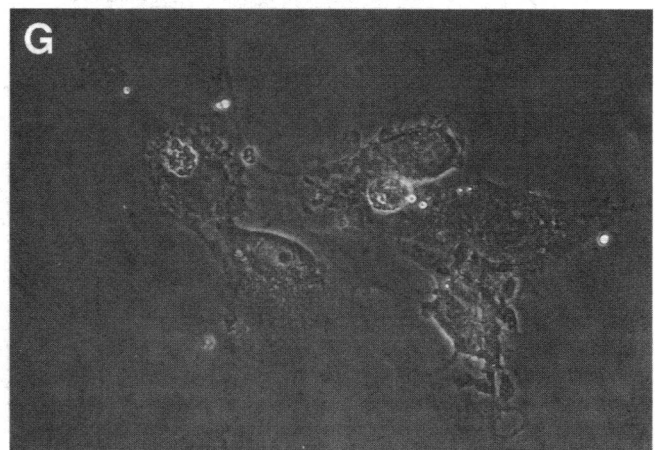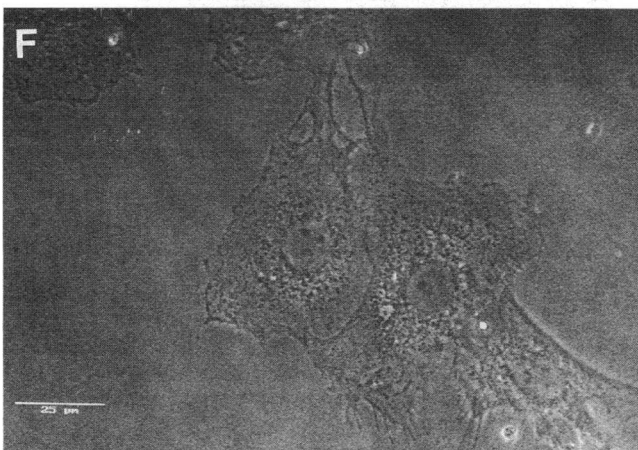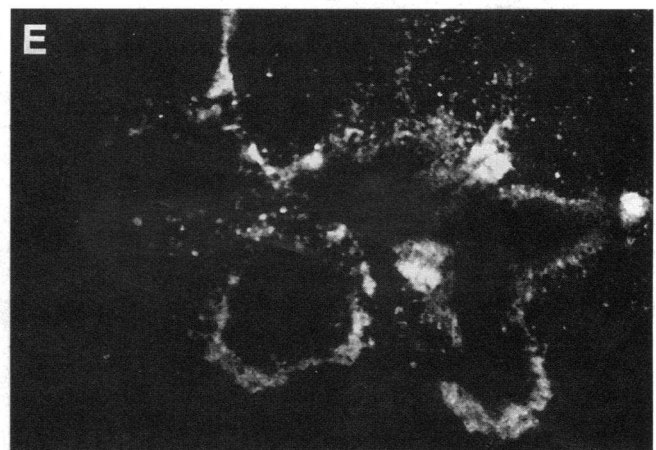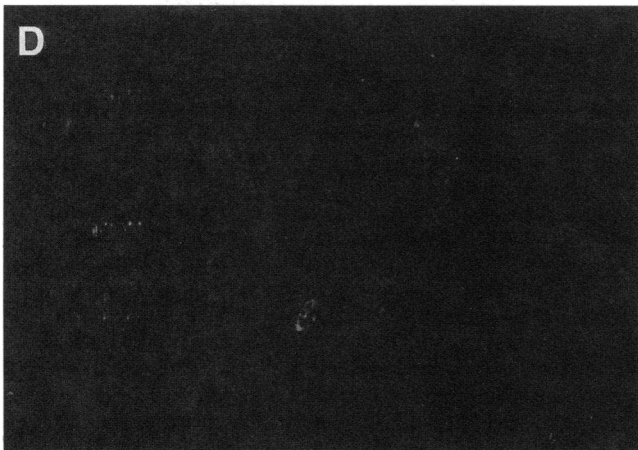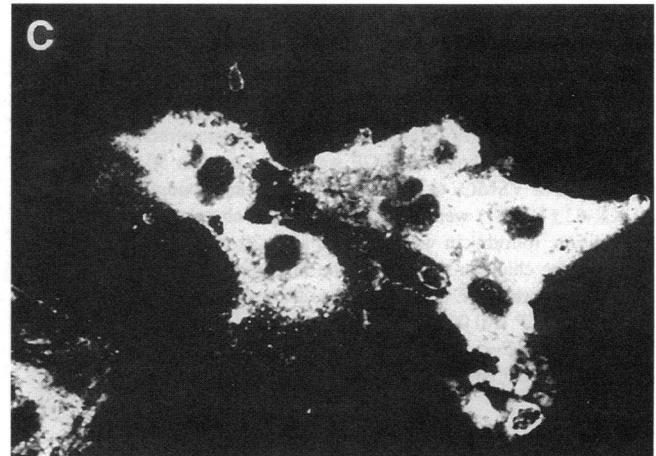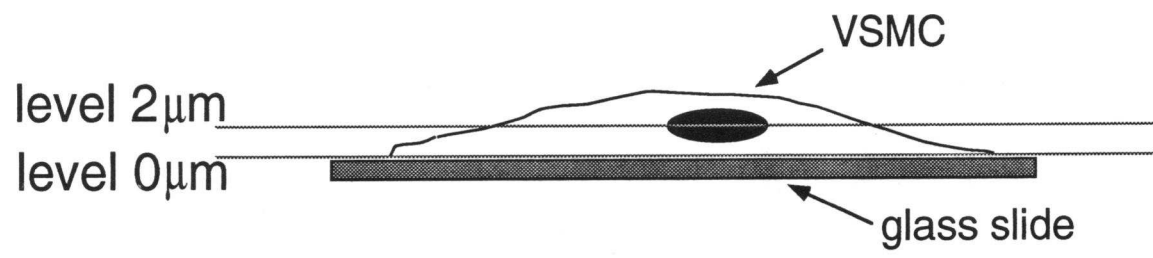
the formation of the neointima after vascular injury has been reported, where it has been directly shown to be an important mediator of medial VSMC replication after vessel injury (13, 14). In addition, bFGF induces angiogenesis (44–46) and stimulates the migration of endothelial cells (47–49). Endogenous bFGF has also been shown to be an essential part of the mechanism by which thromboxane A2 stimulates hypertrophy of VSMCs (24). Evidence that bFGF also affects the migration of VSMCs is supported by data in the literature. In vitro studies have shown that increased migration in response to PDGF occurs within 24 h of wounding a culture of VSMCs, is associated with an elevation in bFGF mRNA that peaks at ∼ 12 h, and is blocked by neutralizing antibodies to bFGF (15). These studies, however, did not distinguish whether bFGF acted as a cofactor to facilitate migration toward PDGF or as a chemoattractant itself. Our studies indicate that bFGF is, at best, a weak chemoattractant for cultured rat VSMCs and that bFGF's role in PDGF-stimulated migration is to facilitate intracellular signaling required for migration.

Other studies indicate that bFGF may play an important role in the migration of VSMCs in vivo in response to vessel injury. In rat carotid arteries that have been deendothelialized with a balloon catheter, there is significantly more medial cell migration and neointima formation when traumatic injury to the underlying smooth muscle cell layers accompanies deendothelialization. Such injury is thought to facilitate migration by causing the release of bFGF from VSMCs when the integrity of their plasma membranes are compromised by the injury. Consistent with this interpretation is the observation that infusion of bFGF-neutralization antibodies into such vessels blocks the increase in medial cell migration and neointima size (13). In addition, the neointimal response to gentle deendothelialization can be increased by systemic injection of bFGF (16). While these data cannot rule out the possibility that bFGF is acting as a VSMC chemoattractant on its own, it is clear that PDGF, which would be expected to be released from activated platelets that accumulate on the surface of the denuded vessel regardless of the extent of underlying medial cell injury, is not sufficient on its own to induce significant medial cell migration. Injury to the underlying media either releases a cofactor or enables cells to respond to a cofactor that is always present, thereby facilitating cell migration. The data we have presented here suggest that that factor may be bFGF. One test of this hypothesis will be to determine whether neutralizing PDGF antibodies block medial cell migration in gently deendothelialized vessels infused with bFGF.

The mechanism by which bFGF affects PDGF signal transduction and enables it to activate CamKinase II is not known. Both PDGF and bFGF interact with cell surface receptors that are ligand-activated tyrosine kinases (50, 51). These kinases autophosphorylate the receptors, facilitating the docking and subsequent activation of a number of intracellular proteins that control different signaling transduction pathways (51). Activation of phospholipase C-γ (PLC-γ) via its docking to the activated receptor would be the most direct pathway from receptor

stimulation to CamKinase II activation. Activation of PLC-γ leads to the the hydrolysis of phosphatidylinositol bisphosphate and the formation of diacylglycerol and inositoltriphosphate (IP₃) (52). IP₃, through its ability to rapidly raise the intracellular calcium level through the release of calcium from intracellular stores (53), then leads to activation of CamKinase II (54). Our measurements of the changes in intracellular calcium levels in response to PDGF demonstrate that the early calcium release attributable to IP₃ was dramatically truncated in bFGF antibody–treated cells (Fig. 8). The addition of ionomycin, which causes a rapid increase in intracellular calcium similar to that seen in proliferating PDGF-stimulated cells (8), activated CamKinase II, and restored the ability of such cells to migrate (Fig. 9). It is unlikely that endogenous bFGF is directly responsible for the release of IP₃-sensitive calcium stores because the changes that occurred in $[Ca^{2+}]_i$ after PDGF stimulation occurred rapidly after the addition of PDGF and during a time in which there was no significant change in bFGF content. In addition, exogenous bFGF on its own neither increases $[Ca^{2+}]_i$ nor stimulates CamKinase II activation or activity (Fig. 6). These results suggest that endogenous bFGF either affects the ability of PDGF to activate PLC-γ, the ability of PLC-γ to synthesize IP₃, the content of calcium in intracellular stores, or the release of those stores by IP₃.

Although the changes in intracellular calcium dynamics seen with antibody treatment currently provide a satisfactory explanation of the data, other possible mechanisms cannot be excluded. For example, bFGF can also activate protein kinase C (PKC) through PLCγ-independent pathways (55, 56). PKC, in turn, can potentiate the actions of CamKinase II by increasing the availability of cytosolic calmodulin (57, 58). Antibodies that block bFGF could, therefore, interfere with CamKinase II activation by ultimately regulating calmodulin levels. Note, however, that downregulating PKC in proliferating VSMCs, thereby blocking the ability of either PDGF or bFGF to activate PKC, has been shown to have no effect on cell migration (8). Ionomycin's ability to restore migration in antibody treated cells, however, might be through an indirect effect on calmodulin levels and not solely through intracellular calcium content, although no such effects have been reported.

An additional complication to understanding the mechanism by which bFGF assists PDGF-directed migration in VSMCs is that bFGF does not contain a signal peptide sequence for secretion (34). This fact and the observations that bFGF can be found in the nuclei of many different cells (59) has led to the suggestion that bFGF may not be required to exit the cell to initiate its effects (60). Evidence for secretion by exocytosis that is independent of the endoplasmic reticulum–Golgi pathway, however, has been presented (61) and the micrographs in Fig. 10 E demonstrate that bFGF is present in the extracellular matrix surrounding VSMCs. Likewise, previous studies on VSMCs have clearly demonstrated the extracellular presence of VSMC-derived bFGF (49). Exogenously applied bFGF can restore the ability of growth-arrested VSMCs to migrate toward

Figure 10. Confocal microscopy of bFGF localization in VSMC cultures. (*A*) Schematic of different planes sampled by the confocal microscope that were parallel to the culture slide. (*B*) and (*C*) Staining of cultured VSMCs with monoclonal bFGF antibody (*C*) or isotype matched control (*B*) through a plane 2 μm above the slide surface and through the cell nucleus (level 2 μm). (*D*) and (*E*) Staining of cultured VSMCs with monoclonal bFGF antibody (*E*) or isotype-matched control (*D*) through the plane of the slide surface (level 0 μm). (*F*) and (*G*) Phase-contrast images of fields shown in *B* and *D* (*F*) and *C* and *E* (*G*) above.

PDGF. This restoration is associated with the ability of these cells to activate CamKinase II in response to PDGF (Bilato, C., R. Pauly, R. Monticone, and M. Crow, unpublished observations), a result consistent with the studies described here and an extracellular mode of bFGF action.

In summary, we have shown that bFGF, which is necessary for both in vivo and in vitro migration of VSMCs, is required for PDGF to complete the intracellular signaling needed for in vitro migration. While multiple intracellular signaling pathways are likely to be required for a complex cellular behavior such as migration, the data presented here reinforce the central role of CamKinase II activation in the regulation of that behavior in VSMCs. The results indicate that multiple growth factors/cytokines may be needed to activate the program of cell migration in VSMCs. This requirement for multiple factors in the activation of migration may explain why migration in vivo occurs only after several days of injury to blood vessels and is dependent on the extent of injury to the underlying media.

Acknowledgments

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