The Inhibition of Vascular Smooth Muscle Cell Migration by Peptide and Antibody Antagonists of the $\alpha_v\beta_3$ Integrin Complex Is Reversed by Activated Calcium/Calmodulin–dependent Protein Kinase II

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Abstract

The migration of vascular smooth muscle cells (VSMCs) is thought to play a key role in the pathogenesis of many vascular diseases and is regulated by soluble growth factors/chemoattractants as well as interactions with the extracellular matrix. We have studied the effects of antibodies to rat β3 and human $\alpha_v\beta_3$ integrins on the migration of VSMCs. Both integrin antibodies as well as cyclic RGD peptides that bind to the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ significantly inhibited PDGF-directed migration. This resulted in a reduction in the accumulation of inositol (1,4,5) trisphosphate and the activation of calcium/calmodulin–dependent protein kinase II (CamKII), an important regulatory event in VSMC migration identified previously. PDGF-directed VSMC migration in the presence of the anti-integrin antibodies and cyclic RGD peptides was restored when intracellular CamKII activity was elevated by either raising intracellular calcium levels with the ionophore, ionomycin, or infecting with a replication-defective recombinant adenovirus expressing a constitutively activated CamKII cDNA (AdCMV.CKIID3). Rescue of rat VSMCs was also observed in stably transfected cell lines expressing constitutively activated but not wild-type CamKII. These observations identify a key intermediate in the regulation of VSMC migration by outside–in signaling from the integrin $\alpha_v\beta_3$. (*J. Clin. Invest.* 1997. 100:693–704.)
Key words: cell migration • vascular smooth muscle cells • integrins • calcium • calcium/calmodulin–dependent protein kinase II

Introduction

The migration of vascular smooth muscle cells (VSMCs)[1] from the tunica media to the intima is considered an important pathogenic event in many vascular disorders such as atherosclerosis and restenosis after balloon angioplasty (1, 2). The majority of VSMCs in the normal arterial wall reside in the tunica media, where they are surrounded by and embedded in a variety of extracellular matrix (ECM) molecules. VSMCs interact with these matrix components, which are likely to serve not only as anchorage sites and barriers to cell movement, but also as regulators of VSMC gene expression and modulators of intracellular signaling pathways initiated by growth factors and/or chemoattractants. For example, the composition of the ECM has been shown to promote the modulation of VSMCs from a contractile to a synthetic phenotype (3, 4), a process required for VSMC migration (5).

Cell–matrix interactions are mediated, in part, by the ubiquitously expressed class of cell surface receptors known as integrins (6). These molecules assemble as heterodimers through the noncovalent association of α and β subunits (7, 8). Both subunits are membrane glycoproteins composed of a large extracellular domain, a single membrane-spanning segment and, with the exception of the β4 subunit, a short cytoplasmic tail. In general, the extracellular region contains the binding site for ECM ligands, while the intracellular portion associates with cytoskeletal elements (9). On the basis of the different β subunit types, the integrins are currently classified into eight subgroups (10). Integrins containing either the β1 or β3 subunits are primarily involved in cell–ECM interactions, while other β subclasses mediate cell–cell interactions. Integrins not only serve as transmembrane links between the ECM and the cell surface resulting in increased adhesion, but they also can trigger important intracellular events (11). The role of integrins in controlling cell migration is likely, therefore, to be multifaceted and involve not only the regulation of adhesion and spreading, but also intracellular signaling events triggered by various chemoattractants. Previous studies have established the presence of various integrins on the cell surface of VSMCs that are responsible for adhesion to the ECM and regulation of cell movement (4, 12–19). Among these, the integrin complex $\alpha_v\beta_3$ is of special interest. It is the most promiscuous of all integrin complexes in terms of its ability to recognize a wide variety of Arg-Gly-Asp (RGD)-containing ECM components (20–23). An important role for this complex in the pathogenesis of various diseases is supported by several observations. First, $\alpha_v\beta_3$ expression is upregulated in several invasive tumors (24, 25). In human melanoma cells, its expression is correlated with malignancy grade (26), collagenase production (27), and cell growth (28). Second, blocking the $\alpha_v\beta_3$ receptor with peptide antagonists or function-blocking antibodies prevents the vascularization of tissues and tumors (29) and reduces tumor size (30). Third, $\alpha_v\beta_3$ expression is upregulated in many intimal lesions associated with the various stages of atherosclerosis (31). In animal models of restenosis, peptide antagonists of $\alpha_v\beta_3$ effectively block the fibroproliferative response seen in

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1. *Abbreviations used in this paper:* CamKII, calcium/calmodulin protein kinase II; cRGD, cyclic RGD; ECM, extracellular matrix; HPF, high power field; IP3, inositol (1,4,5) trisphosphate; PI(4,5)P2, phosphoinositol (4,5) bisphosphate; PLCγ, phospholipase Cγ; PMSF, phenylmethylsulfonylfluoride; TSP, thrombospondin; VSMCs, vascular smooth muscle cells; WT, wild type.

blood vessels after balloon catheter injury (32, 33). Moreover, there is growing evidence that the long term beneficial effects of a humanized β_3 integrin antibody on survival after balloon angioplasty in humans (34) might be due, in part, to its ability to inhibit both $\alpha_{IIb}\beta_3$ present on platelets and the $\alpha_v\beta_3$ complex present on other vascular cells.

Multiple intracellular signaling events control the directed migration of VSMCs toward the chemoattractant, platelet-derived growth factor (PDGF) (35, 36). We have demonstrated previously that the activation of the multifunctional protein kinase, calcium/calmodulin–dependent protein kinase II (CamKII), is a critical signaling event for VSMC migration. This enzyme is regulated by the phenotypic state of the cell (37) and the cooperation between PDGF and basic fibroblast growth factor (bFGF or FGF-2), which is synthesized by VSMCs in response to PDGF (38). Here we report that occupancy of $\alpha_v\beta_3$ is also required for the PDGF-induced migration of both rat and human VSMCs and that the inhibition of migration caused by cyclic RGD (cRGD) peptides or $\beta_3/\alpha_v\beta_3$ integrin antibodies is associated with a failure of PDGF to fully activate CamKII. When CamKII was activated independently of PDGF either by elevating intracellular calcium with an ionophore or through forced expression of a recombinant-engineered constitutively activated CamKII, the antagonists were no longer effective in suppressing migration. Our results demonstrate that PDGF-directed migration in VSMCs requires integrin-mediated intracellular events that regulate chemoattractant-activation of the multifunctional protein kinase, CamKII.

Methods

Materials. Recombinant human PDGF-BB (Collaborative Research Inc., Lexington/Waltham, MA or Upstate Biotechnology, Inc., Lake Placid, NY) was dissolved in Dulbecco's modified MEM (high glucose; GIBCO BRL, Gaithersburg, MD) containing 0.1% BSA, aliquoted, and stored at −70°C. Calmodulin (Upstate Biotechnology, Inc.) and autocamtide (Peninsula Laboratories Inc., Belmont, CA) were dissolved in water, aliquoted, and stored at −20°C. All reagents were used only once after thawing. cRGD (GpenGRGDSPCA), RGD (GRGDSP), and RGE (GRGESP) peptides were purchased from GIBCO BRL. Cyclo-RGDFV was purchased from Peptides International (Lexington, KY). All peptides were freshly prepared for each experiment by reconstitution in water. cRGD specifically recognizes the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and has no effect on other integrins (12, 39). Monoclonal antibody F11 (40), directed against rat β3–containing integrins, was generously provided by Dr. Michael Horton (University College, London) or purchased from PharMingen (San Diego, CA). Monoclonal antibodies LM609 and P1F6, directed against the human $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, respectively, were provided generously by Dr. David Cheresh (Scripps Institute, La Jolla, CA) or purchased from Chemicon International, Inc. (Temecula, CA). Stably transfected cell lines of rat aortic medial VSMCs expressing full-length wild type (WT) and constitutively activated (D3 mutation) for rat brain CamKII α-subunit have been described previously and characterized (37).

Cell cultures. Medial VSMCs were enzymatically dissociated from the thoracic aorta of 3–6-mo-old Wistar rats as described previously (5). Cells were maintained in culture with Dulbecco's modified MEM (high glucose) supplemented with 10% heat-inactivated FBS, 1 mmol/liter nonessential amino acids, 20 mmol/liter L-glutamine, 50 μg/ml streptomycin, and 10 μg/ml neomycin (GIBCO BRL) in a humidified 5% CO_2 atmosphere at 37°C. Human aortic VSMCs were purchased from Clonetics (San Diego, CA) and grown in smooth muscle growth medium-2 (SMGM-2) supplemented with 5% FBS and 5 μg/ml insulin. 2 μg/ml human FGF, 0.5 ng/ml human EGF, 0.5 μg/ml gentamicin, and 0.5 μg/ml amphotericin (all purchased from Clonetics). For rat VSMCs, proliferating cells were used between the 7th and 14th passages and for human VSMCs between the 5th and 7th passages. Cells were harvested for the migration/chemotaxis assay or biochemical analyses between 50 and 80% confluence. The transformed human embryonic kidney cell line 293 (ATCC CRL 1573) (American Type Culture Collection, Rockville, MD) was grown in improved minimal essential medium (I-MEM) (Biofluids, Inc., Rockville, MD) containing 10% FBS, 2 mmol/liter glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Chemotaxis/migration assay. Chemotaxis/migration assays were performed in a modified Boyden chamber as described previously (5, 41). Polycarbonate filters (Nucleopore polycarbonate, 8 μm pores; Costar Sci. Corp., Pleasanton, CA) were coated overnight with 50 μg/ml of either human or rat fibronectin (GIBCO BRL). A solution of 0.4 nmol/liter PDGF-BB diluted in Dulbecco's modified MEM (high glucose) and 0.1% BSA was placed in the bottom chamber of the Boyden apparatus. Cells (200,000) suspended in Dulbecco's modified MEM (high glucose) and 0.1% containing 0.1% BSA were then added to the upper chamber in a final volume of 0.8 ml. Other reagents were used at the concentrations indicated and either added to the upper chamber at the time of cell seeding or preincubated with the cells for 30 min before cell seeding of the chambers. The distinction between chemotaxis and chemokinesis was evaluated as described previously (38). When adhesion of the cells on the coated filter was assessed, only 50,000 VSMCs were plated and the number of cells on the top surface of the filter was calculated at the end of the 4 h experiment. In all cases, four fields per filter were counted and all experiments were run in triplicate. Each triplicate assay was repeated at least three times on separate occasions with different VSMC preparations.

Cell adhesion assay. The individual wells of a 96-well microtiter plate (Nunc, Inc., Naperville, IL) were coated overnight at 4°C with 10–50 μg/ml of purified fibronectin, vitronectin, or thrombospondin dissolved in Dulbecco's PBS. Nonspecific binding sites were blocked with 5 mg/ml denatured BSA for 1 h at 37°C. Peptides and antibody antagonists were added to the cell suspension before plating. Cells resuspended in Dulbecco's modified MEM and 1% BSA were added to the coated wells (30,000 cells per well) and adhesion at 37°C for 1 h was measured by first rinsing away nonadherent cells with Dulbecco's PBS followed by fixation of adherent cells with 4% formaldehyde in PBS. The plates were then stained with 0.5% toluidine blue in 4% formaldehyde for 5 min and then rinsed extensively with water. The stain was eluted from the adherent cells by incubating in 1% SDS for 15 min at room temperature and then quantified at 595 nm.

CamKinase II activation and activity assays. Since CamKII undergoes regulatory autophosphorylation (42), the activation of the enzyme in response to PDGF-BB can be evaluated by following the incorporation of radioactive phosphate into the molecule. For this measurement, proliferating VSMCs at 70–80% confluency were incubated in phosphate-free medium containing 0.3 mCi/ml [^{32}P]orthophosphoric acid for 3–5 h and then stimulated with PDGF-BB in the presence or absence of cRGD peptide or antibody, F11. The cells were then extracted and prepared for immunoprecipitation as described previously (37). Immunoprecipitation was carried out using an antibody (generous gift of Dr. Harold Singer, Weis Center for Research, Danville, PA) against the δ-subunit of CamKII, which is the predominant isoform expressed by rat VSMCs (37, 43). Equivalent amounts of TCA-precipitable counts were separated by SDS-PAGE on a 10% gel, which was then dried and exposed for autoradiography. The incorporation of ^{32}P into CamKII was quantified using a PhosphorImager (Betascope Model 603; Betagen Corp., Waltham, MA).

CamKII activity in cellular extracts was measured by monitoring phosphorylation of a synthetic CamKII substrate (autocamtide-2) in the presence (calcium-dependent activity) or absence (calcium-independent activity) of calcium and calmodulin (37). The autonomous activity is expressed as the percentage of the calcium-independent activity relative to total activity.

Recombinant adenovirus vector construction. A replication-deficient adenovirus in which the majority of the E1 region had been deleted and replaced with a cassette containing a site for gene insertion and the cytomegalovirus (CMV) early promoter was used. A cDNA for a constitutively active mutant of CamKII (known as CKIID3), generated by site-directed mutagenesis of amino acids 286 and 287 to aspartic acid, was placed downstream of the CMV promoter. The mutations mimic the negative charge introduced in this region by the phosphorylation of the wild-type molecule, resulting in a constitutively activated enzyme (44). AdCMV.CKIID3 was produced by first inserting the CKIID3 cDNA into the pS5 shuttle vector to generate pS5CMV.CKIID3. This was then transfected into 293 cells along with the plasmid pJM17 (Microbix Inc., Toronto, Canada) that contains the full sequence of E1-deleted Ad 5 DNA (45) and individual plaques arising from homologous recombination were isolated. AdCMV.CKIID3 was propagated in 293 cell cultures, purified by CsCl density centrifugation (46), dialyzed, and stored at −70°C in 10 mM Tris-HCl, pH 7.4/1 mM $MgCl_2$ buffer. The titer of the viral stock was determined by plaque assay using 293 cells (47). An adenovirus expressing the *Escherichia coli* β-galactosidase gene (AdCMV.gal) was used as a control for infection.

AdCMV.CKIID3 and AdCMV.gal infection of VSMCs. Cultured VSMCs at 70–80% confluency were exposed to recombinant adenovirus at an moi of 100 in a minimal volume of Dulbecco's modified MEM (high glucose) for 1 h. Additional media containing 10% FBS was then added and the culture incubated at 37°C in 5% CO_2 atmosphere. After 24 h, the virus-containing medium was replaced with virus-free medium and the cells collected for analysis of expression or for migration assays. Under the conditions described above, no cytopathic or cytotoxic effects were observed and > 90% of the cells were infected, as demonstrated by staining the AdCMV.gal–infected VSMCs with X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside; Gold Biotechnology Inc., St. Louis, MO) or the AdCMV.CKIID3–infected VSMCs by immunocytochemistry using an α-Camkinase II antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN). Expression of CKIID3 was also monitored by Northern and Western blotting. Total RNA was isolated by the guanidinium isothiocyanate procedure (48) from cultures of VSMCs at different times after adenoviral infection and probed by Northern blotting analysis as described previously (7). A full-length CamKII cDNA probe generated by PCR and cloned into the pCRII vector (Invitrogen Corp., La Jolla, CA) was verified by dideoxy DNA sequencing. The rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe has been described elsewhere (38). For Western blotting analysis, infected cells were extracted in RIPA buffer (150 mmol/liter NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mmol/liter Tris HCl, pH 8.0, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonylfluoride (PMSF) and clarified by centrifugation and assayed for protein content. 20 μg of protein per sample was separated by SDS-PAGE on a 10% gel and transferred to a polyvinylidene difluoride (PVDF) membrane with a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, CA) at 170 mA for 2 h. The membrane was blocked for 1 h in 5% nonfat milk/PBS (blocking solution) and then incubated overnight at 4°C with a CamKII α-isoform–specific mouse monoclonal antibody (10 μg/ml) (Boehringer Mannheim Biochemicals). The membrane was incubated with a horseradish peroxidase–conjugated anti–mouse antibody (1 μg/ml) for 4 h at 4°C. The membrane was rinsed with PBS and antigen–antibody complexes were detected using enhanced chemiluminescence (ECL) (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

IP3 levels. IP3 levels in VSMCs were determined with a commercially available IP3 receptor binding assay kit (Amersham Corp.). VSMCs were plated on 100 mm tissue culture plates so that the final cell density after an overnight incubation in serum-containing media followed by 48 h of serum starvation was between 0.7 and 1.3×10^6 cells per plate. Cells were exposed to 1 mg/ml cRGD peptide for 30 min before PDGF-BB stimulation. Stimulation was terminated by placing the plates on ice, removing the serum-free media, and adding 0.5 ml of 4% perchloric acid. Each sample was collected by scraping the cells and precipitated proteins into a 1.5 ml centrifuge tube. The extract was placed on ice for 20 min and then clarified by centrifugation at 4°C. The supernatant was neutralized to approximately pH 7.5 with 1.5 N KOH/60 mM Hepes buffer, pH 8. The pellet was dissolved in 0.3 N NaOH and protein content determined using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). IP3 levels in 0.1-ml aliquots of the neutralized supernatants were assayed with a competitive IP3 receptor binding radioassay according to the manufacturer's instructions. Nonspecific binding was determined by the addition of excess unlabeled IP3 to the assay, while total binding for each assay was determined as the amount of labeled IP3 bound in the absence of competitor. A standard curve was established using serial dilutions of purified IP3. IP3 content in the extracts was determined by interpolation of the standard curve using the curve fitting program, AssayZap (Biosoft Co., Ferguson, IL) and was expressed relative to total protein content.

Tyrosine phosphorylation of PLCγ. After pretreatment and stimulation, VSMCs were rinsed in ice-cold Dulbecco's PBS and then extracted by scraping the cells into 0.5 ml/100 mm plate of ice-cold lysis buffer (0.5 M Tris, pH 8.0, 137 mM NaCl, 1 mM Na_3VO_4, 1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, and 10% glycerol). The extract was placed on ice for 30 min and then clarified by centrifugation at 4°C. Supernatants containing equal amounts of protein were adjusted to a final volume of 1 ml using wash buffer (20 mM Tris, pH 8, 137 mM NaCl, 1 mM PMSF, and 10 μg/ml aprotinin). Immunoprecipitation of PLCγ was performed overnight at 4°C with 1 μg/ml of mixed monoclonal antibodies to bovine PLCγ (Upstate Biotechnology Inc.). Protein A-Sepharose (50 μl of a 50% slurry) (Sigma Chemical Co.) was added to each sample, which was then incubated with mixing at 4°C for another hour. Immunocomplexes were pelleted by 30 s centrifugation in a microfuge and the pellet washed with 1 ml of each of the following solutions: (*a*) 1% NP40, 150 mM NaCl, 20 mM Tris, pH 8.0, 10% glycerol, 2 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM PMSF; (*b*) 0.5% NP40, 0.5 M LiCl, 50 mM Tris HCl, pH 7.5; (*c*) 0.5 M LiCl, 50 mM Tris HCl, pH 7.5; and (*d*) 10 mM Tris HCl, pH 7.5. The pellet was resuspended in 50 μl 2× sample buffer (63 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.05% 2-mercaptoethanol, and 0.02% glycerol), boiled for 5 min, and then separated by SDS-PAGE on a 6% gel. After separation, proteins were electrophoretically transferred to a PVDF membrane for 4 h at 170 mA using a semidry blotting apparatus. The membrane was blocked overnight at 4°C in PBS containing 0.1% Tween 20 and 1% BSA and then washed three times for 5 min each at room temperature in Western buffer (50 mM NaCl, 10 mM Tris, pH 7.0, 1 mM EDTA, and 0.1% Tween 20). This was followed by incubation of the membrane in 0.5 mg/ml HRP-conjugated antiphosphotyrosine (ICN Biomedicals, Inc., Irvine, CA) for 2 h at room temperature. The membrane was washed in Western buffer at room temperature and the reactive bands on the membrane visualized by chemiluminescence according to the manufacturer's instructions (Amersham Corp.). Band intensity was quantitated using a densitometer (Molecular Dynamics, Sunnyvale, CA).

Statistical evaluations and comparisons. All the data are expressed as the mean±SEM. The comparison of the mean values among the different groups was made by ANOVA with *P* value corrected by the Bonferroni method (49).

Results

An antibody to rat β3 integrins and cyclic RGD peptides block rat VSMC chemotaxis. Fig. 1 summarizes the results of experiments showing the effects of a rat β3 integrin antibody (F11) or a cRGD peptide (GpenGRGDSPCA) on the chemotaxis of VSMCs toward 0.4 nM PDGF BB. In untreated cultures, PDGF BB consistently stimulated migration more than eightfold above BSA controls (BSA alone, 5.3±3.3 cells per high

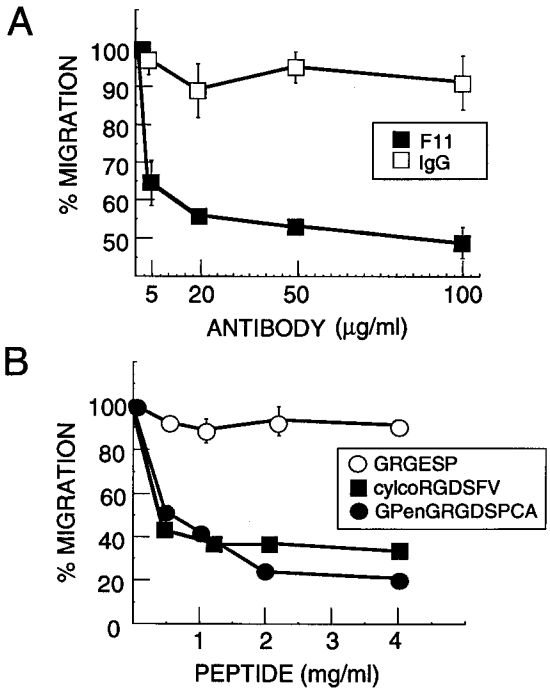

Figure 1. β3 integrin antibody and cyclic RGD peptides inhibit PDGF-directed chemotaxis in rat VSMCs. Boyden chamber chemotaxis assays of cultured rat medial VSMCs toward BSA or 0.4 nM PDGF-BB in the presence of varying doses of the β3 integrin antibody, F11 (*A*) or the cRGD peptides, GPenGRGDSPCA and cyclo-RGDFV (*B*). Results represent at least three independent experiments and are expressed relative to chemotaxis/migration in the absence of antibody or peptide after subtraction of BSA background. (BSA: 5.25±1.3 cells per HPF; PDGF: 81.3±4.9 cells per HPF).

power field [HPF] PDGF-BB, 81.3±4.9 cells per HPF). When the rat β3 integrin antibody F11 was added to the upper chamber, there was a dose-dependent reduction in PDGF-mediated migration, with a half-maximal effect at < 5 μg/ml (Fig. 1 *A*). In contrast, equivalent amounts of mouse IgG had no effect on PDGF-directed chemotaxis.

Likewise, when the cRGD peptides, GPenGRGDSPCA and cyclo-RGDFV, were each added to the upper chamber, there was also a dose-dependent reduction in PDGF-directed migration. As shown in Fig. 1 *B*, there was an $\sim 50\%$ reduction in PDGF-directed chemotaxis seen with 0.5 mg/ml of either cRGD peptide. In contrast, the addition of equivalent amounts of the control RGE peptide, GRGESP, did not affect the PDGF-directed migration of rat VSMCs. Although further inhibition of chemotaxis was observed when the dose of the GPenGRGDSPCA peptide was increased above 2 mg/ml, this high dose inhibition was not seen with cyclo-RGDFV. Even at these high doses, the cRGD peptides did not interfere with either cell adhesion or spreading on the substrates used to coat the filters. When the cells on the top surface of the filter were counted after the 4 h incubation period, there was no significant difference in cell attachment between cRGD peptide-treated and untreated cells (68.1±13.1 versus 69.6±13.9). Adhesion was also measured using a microplate assay in which different ECM components were absorbed to polyvinylchloride plates (Fig. 2). These results show no change in attachment of cells to fibronectin as a result of preincubation with cRGD, RGE, or the β3 integrin antibody, F11. As expected, attachment of cRGD-treated but not RGE-treated cells was inhibited on vitronectin-coated plates, indicating that the cyclic peptide does interfere with ECM-vitronectin receptor interactions. F11 also did not affect attachment to fibronectin. Unlike the cRGD peptides, F11 also did not alter adhesion to vitronectin, probably because vitronectin-binding integrin complexes, such as $\alpha_v\beta_5$, are unaffected by F11. These results show that while both F11 and the cRGD peptides interfere with PDGF-directed chemotaxis, this is not a consequence of the inability of the treated cells to attach to the ECM-coated membrane used in the Boyden apparatus. The fact that both the cRGD peptide, which engages α_v-containing integrins, and the F11 antibody, which recognizes only β3-containing integrin complexes, inhibit PDGF-directed chemotaxis to the same extent in these experiments suggests that the target of these reagents is probably $\alpha_v\beta_3$. This can be directly tested in human VSMCs where antibodies to specific human integrin complexes are available.

As was the case for rat VSMCs, the number of human VSMCs migrating to the underside of the membrane was markedly higher when the cells were exposed to a PDGF-BB gradient compared to BSA alone (85.5±2.5 versus 8.3±0.5; data not shown). Preincubation with 1 mg/ml cRGD peptide reduced the migration of these cells by $> 50\%$ relative to cells exposed to PDGF-BB alone. Monoclonal antibodies directed against specific integrins were then used to identify which integrins were responsible for the inhibitory effect of the cRGD peptide. The monoclonal antibody LM609 is specifically directed against the integrin, $\alpha_v\beta_3$. Treatment of migrating cells with this antibody resulted in a dose-dependent inhibition of migration of up to 60%. Treatment with either a monoclonal antibody directed against $\alpha_v\beta_5$ (PIF6) or with an isotype-matched nonimmune mouse IgG (IgG1) had no effect on PDGF-directed migration (Fig. 3 *A*). The combination of LM609 and P1F6 produced no additional effect other than that

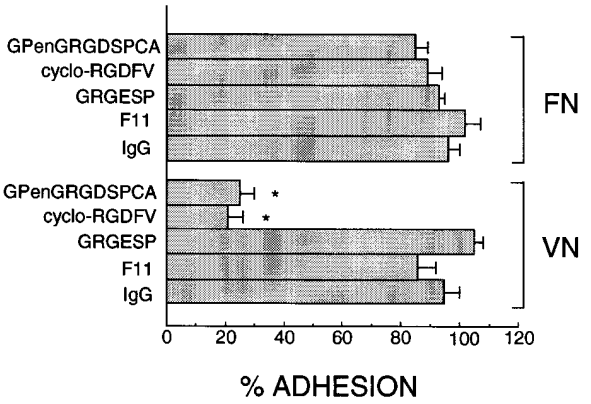

Figure 2. Effect of the β3 antibody F11, cRGD, and RGE peptides on rat VSMC adhesion to fibronectin and vitronectin. Adhesion of cultured rat medial VSMCs was measured using a microtiter plate assay with the individual wells of the plate coated with 50 μg/ml of each of the indicated substrates. Adhesion was allowed to occur for 1 h at 37°C. Nonspecific binding was blocked with 5 mg/ml denatured BSA and was subtracted from all adhesion data. Data are expressed relative to that observed in the absence of either peptide. *FN*: fibronectin; *VN*: vitronectin.

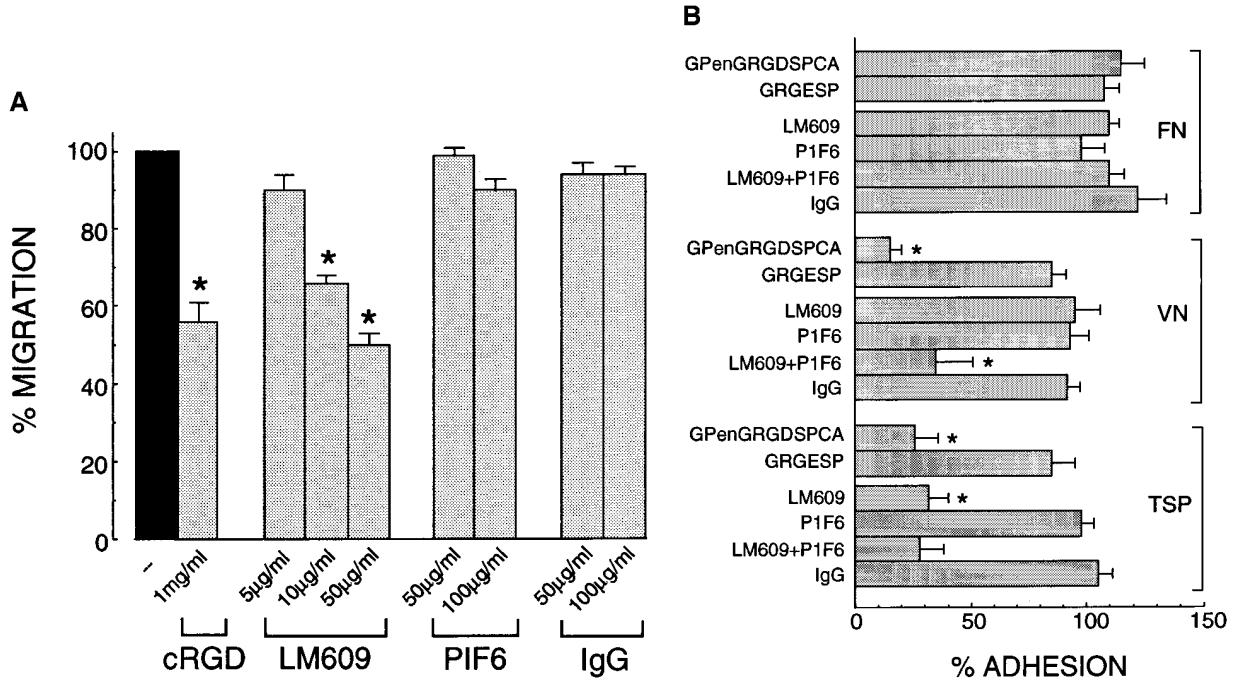

Figure 3. Inhibition of PDGF-directed chemotaxis/migration of human VSMCs by cRGD peptide or monoclonal antibody, LM609. (*A*) The effects of 1 mg/ml cRGD (GPenGRGDSPCA), the $\alpha_v\beta_3$ neutralizing monoclonal antibody, LM609, the $\alpha_v\beta5$ neutralizing monoclonal antibody, P1F6, and nonimmune serum (IgG1) on human VSMC chemotaxis toward PDGF-BB. Results represent at least five independent experiments and are expressed relative to the values for PDGF alone. BSA background: 8.3±0.5 cells per HPF; PDGF control: 85.5±2.5 cells per HPF. *Significantly different from PDGF control value, $P < 0.001$. (*B*) Effects of cRGD peptide and monoclonal antibodies to integrin complexes on the adhesion of human VSMCs to fibronectin, vitronectin, and thrombospondin. Adhesion was measured using a microtiter plate assay with the individual wells of the plate coated with 50 μg/ml of each of the indicated substrates. Adhesion was allowed to occur for 1 h at 37°C. Nonspecific binding was blocked with 5 mg/ml denatured BSA and was subtracted from all adhesion data. Data are expressed relative to that observed in the absence of either peptide. *Significantly different from untreated value, $P < 0.001$. *FN*, fibronectin; *VN*, vitronectin.

seen with LM609 alone (data not shown). These results demonstrate that the integrin complex regulating PDGF-directed migration in human VSMCs was likely to be $\alpha_v\beta_3$. As was the case for rat VSMCs, none of the antibodies or peptides used to block human VSMC chemotaxis affected the ability of human VSMCs to attach to fibronectin. While cRGD was effective in blocking binding of human VSMCs to vitronectin, only a combination of the monoclonal antibodies, LM609 and P1F6, produced any significant blockade of adhesion to vitronectin alone (Fig. 3 *B*). This is consistent with results published previously which indicate that attachment of cells to vitronectin can occur through both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (50, 51). However, LM609 alone was able to block attachment of the human VSMCs to thrombospondin (TSP) while P1F6 was ineffective (Fig. 3 *B*).

PDGF-stimulated CamKinase II activation is suppressed in VSMCs treated with cRGD peptides and β3-integrin antibodies. While numerous intracellular signaling pathways have been implicated in the control of cell migration, a critical role for the multifunctional protein kinase CamKII in VSMC migration has been demonstrated recently (37, 38). To determine if antagonists of the vitronectin receptor affect the ability of VSMCs to activate CamKII in response to PDGF-BB, we measured its activation in treated cells. Activation of CamKII by calcium and calmodulin occurs as a result of autophosphorylation, resulting in an enzyme whose catalytic activity is no longer dependent on calcium and calmodulin. The activity of this enzyme can, therefore, be measured by either monitoring the incorporation of radiolabeled phosphate into the molecule or by measuring the ability of the enzyme to phosphorylate a synthetic peptide substrate in permeabilized cells. Fig. 4 *A* shows the results of monitoring autophosphorylation of the enzyme. After 1 min of incubation with 10 ng/ml PDGF-BB, phosphorylation of CamKII was increased significantly. When VSMCs were pretreated with 1 mg/ml of cRGD peptide for 30 min before the addition of PDGF, there was an ~ 50% decrease in ^{32}P incorporation. Similar results were obtained when we tested the activity of the enzyme using a synthetic substrate in permeabilized cells (Fig. 4 *B*). In this case, autonomous activity refers to enzyme that is active in the presence of EGTA and in the absence of additional calmodulin. It is reported as the percentage of total activity, which is obtained in parallel cultures in which excess calcium and calmodulin was added to the permeabilization solution. Unstimulated VSMC cultures showed a basal level of autonomous activity of 8.9±1.4%. The addition of PDGF-BB resulted in a significant increase of CamKinase II–independent activity within the first minute (58.5±1.8% autonomous activity). Blocking vitronectin receptors with 1 mg/ml cRGD peptide significantly suppressed PDGF-stimulated CamKII activation by ~ 53% (Fig. 4 *B*). Similar results were observed when VSMCs were pretreated with the β3-integrin antibody, F11 (data not shown).

Ionomycin and basic FGF restore PDGF-directed chemotaxis in cRGD peptide- and β3 integrin antibody–treated VSMCs. We have shown previously that elevating intracellu-

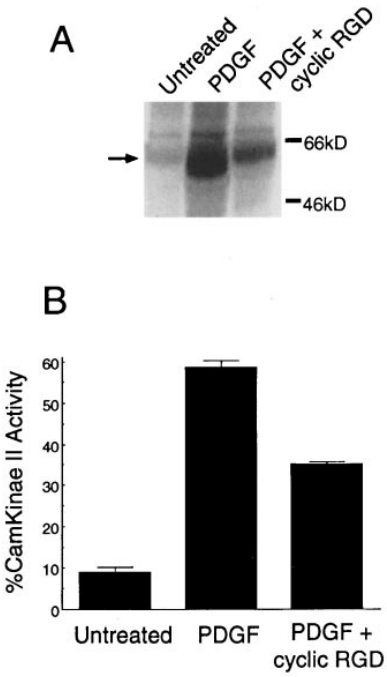

Figure 4. CamKII activation and activity by PDGF-BB is inhibited by cRGD. (*A*) Activation of CamKII measured by ^{32}P incorporation into cellular CamKII. Rat VSMCs were incubated with $^{32}PO_4$, treated, and then extracted with RIPA buffer 1 min after PDGF-BB stimulation. The extract was then incubated with an antisera against δCamKII and immunoprecipitated with protein A–Sepharose. The immunoprecipitate was separated by SDS-PAGE and the gel treated for autoradiography to detect ^{32}P incorporation. The *arrow* indicates the position of a purified CamKII standard, migrating at ~ 53 kD. (*B*) CamKII activity was measured 1 min after 10 ng/ml PDGF-BB stimulation. The activity is expressed as percent autonomous activity, which describes the percent of total activity present in the permeabilized cell extract that is independent of calcium and calmodulin.

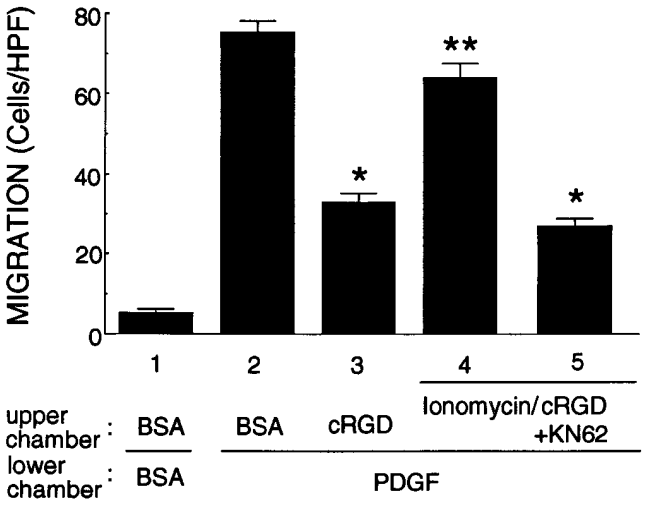

Figure 5. Ionomycin prevents the inhibition of PDGF-directed migration by cRGD peptide. PDGF-directed migration of rat VSMCs. Bar *1*: migration in the absence of a PDGF gradient (6.5±0.4 cells per HPF); bar *2*, migration toward 10 ng/ml PDGF-BB in the lower chamber (78.2±3.1 cells per HPF); bar *3*, migration toward PDGF-BB in the bottom chamber and 1 mg/ml of the cRGD peptide, GPenGRGDSPCA, in the upper chamber (32.7±2.6 cells per HPF); bar *4*, same conditions as in bar *3* but with 1 μM ionomycin also in the upper chamber (65.6±3.6 cells per HPF); bar *5*, same conditions as in bar *4* with 10 μM KN62 also in the upper chamber (25±2.5 cells per HPF). Results are averaged data from four independent experiments. *Indicates statistically significant from PDGF alone (bar *2*), $P < 0.001$; **indicates not statistically significant from PDGF alone (bar *2*).

lar free calcium levels with ionomycin activates CamKII independently of PDGF and restores migration in differentiated cells (37) or in cells with sequestered autocrine factors (38). Given that the cRGD peptide and β3 integrin antibody suppresses CamKII activation in response to PDGF, we determined whether ionomycin could restore migration in antagonist-treated cells (Fig. 5). Proliferating rat VSMCs were placed in the upper chamber of the Boyden apparatus and their ability to migrate was tested in presence or absence of cRGD peptide and ionomycin. The data in Fig. 5 shows that the inhibition of migration resulting from incubation with 1 mg/ml of cRGD peptide was reversed completely by the inclusion of 1 μM ionomycin. KN62, a specific inhibitor of CamKII activation (52), completely blocked the ability of ionomycin to reverse the effects of cRGD. Together, these results demonstrate that elevating intracellular calcium levels independently of PDGF-BB can fully restore the migration of proliferating VSMCs that are inhibited by cRGD and that this restoration is due, at least in part, to the activation of CamKII.

In addition to ionomycin, we have shown previously that addition of bFGF (FGF-2) can restore PDGF-directed chemotaxis to cells when CamKII activity is suppressed by growth arrest/differentiation (38). FGF-2 alone is neither a chemoattractant for VSMCs nor a stimulus for CamKII activation or activity (38). To determine whether the effect of FGF-2 is upstream or downstream of the β3-integrin effect on PDGF-directed chemotaxis, the migration of F11-treated rat VSMCs or LM609-treated human VSMCs was measured in the presence and absence of FGF-2. Fig. 6 shows that FGF-2 fully restored PDGF-directed migration to both rat and human VSMCs treated with cRGD peptides or the integrin antibodies and that this restoration was inhibited by KN62, consistent with our previous study on bFGF and migration (38). These results demonstrate that the rescue of migration by bFGF occurs downstream of the integrin effect and upstream of CamKII activation.

Forced expression of constitutively activated CamKII bypasses the effect of cRGD peptides and β3 integrin antibodies on VSMC chemotaxis. Both ionomycin and FGF-2 are likely to activate other signaling pathways in the cell. To determine if the activation of CamKII alone is sufficient to restore migration in VSMCs treated with cRGD peptides and β3 integrin antibodies, VSMCs were infected with a recombinant replication-defective adenovirus (AdCMV.CKIID3) expressing CamKII that is locked into the activated state by site-directed mutagenesis. When used at an moi of 100, it was routinely possible to demonstrate successful infection with AdCMV.CKIID3 into at least 90% of VSMCs without any accompanying cytotoxic effects (data not shown). Increased steady levels of mRNA expression for the exogenous CamKII in rat VSMCs after infection are shown in Fig. 7 *A*. Since the majority of the endogenous CamKII in VSMCs is δ-isoform, the expression of the exogenous CamKII mutant protein, which was constructed from an α-isoform cDNA, can be monitored with an α-isoform–specific antibody (Fig. 7 *B*). Expression of the mutant CamKII protein was seen as early as 6 h after infection and continued to increase over the next 24 h. Similar results were

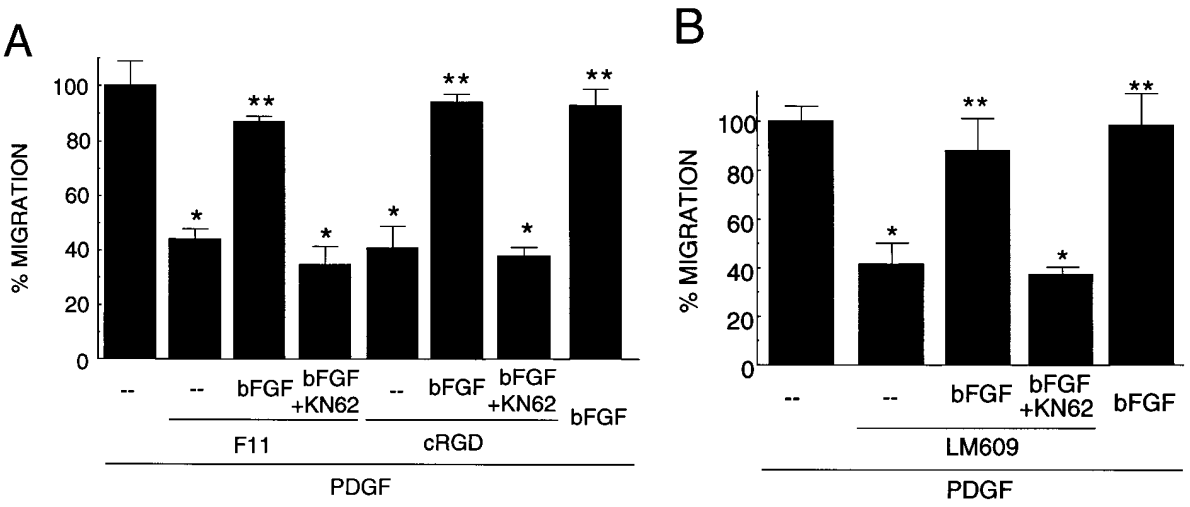

Figure 6. Exogenous bFGF prevents the inhibition of PDGF-directed migration by cRGD peptide. (*A*) PDGF-directed migration of rat VSMCs. Migration expressed relative to that toward 0.4 nM PDGF-BB in the absence of any additional treatments. F11 antibody was added to the upper chamber at a final concentration of 25 μg/ml, while the cRGD peptide, GPenGRGDSPCA, was added at a final concentration of 1 mg/ml. Basic FGF (FGF-2) and KN62 was added to the upper chamber at final concentrations of 10 ng/ml and 10 μM, respectively. Results are averaged data from four independent experiments. *Statistically significant from PDGF alone, $P < 0.001$; **not statistically significant from PDGF alone). (*B*) PDGF-directed migration of human VSMCs. Migration expressed relative to that toward 0.4 nM PDGF-BB in the absence of any additional treatments. LM609 antibody was added to the upper chmaber at a final concentration of 50 μg/ml. Basic FGF (FGF-2) and KN62 was added to the upper chamber at final concentrations of 10 ng/ml and 10 μM, respectively. Results are averaged data from four independent experiments. *Statistically significant from PDGF alone, $P < 0.001$; **not statistically significant from PDGF alone.

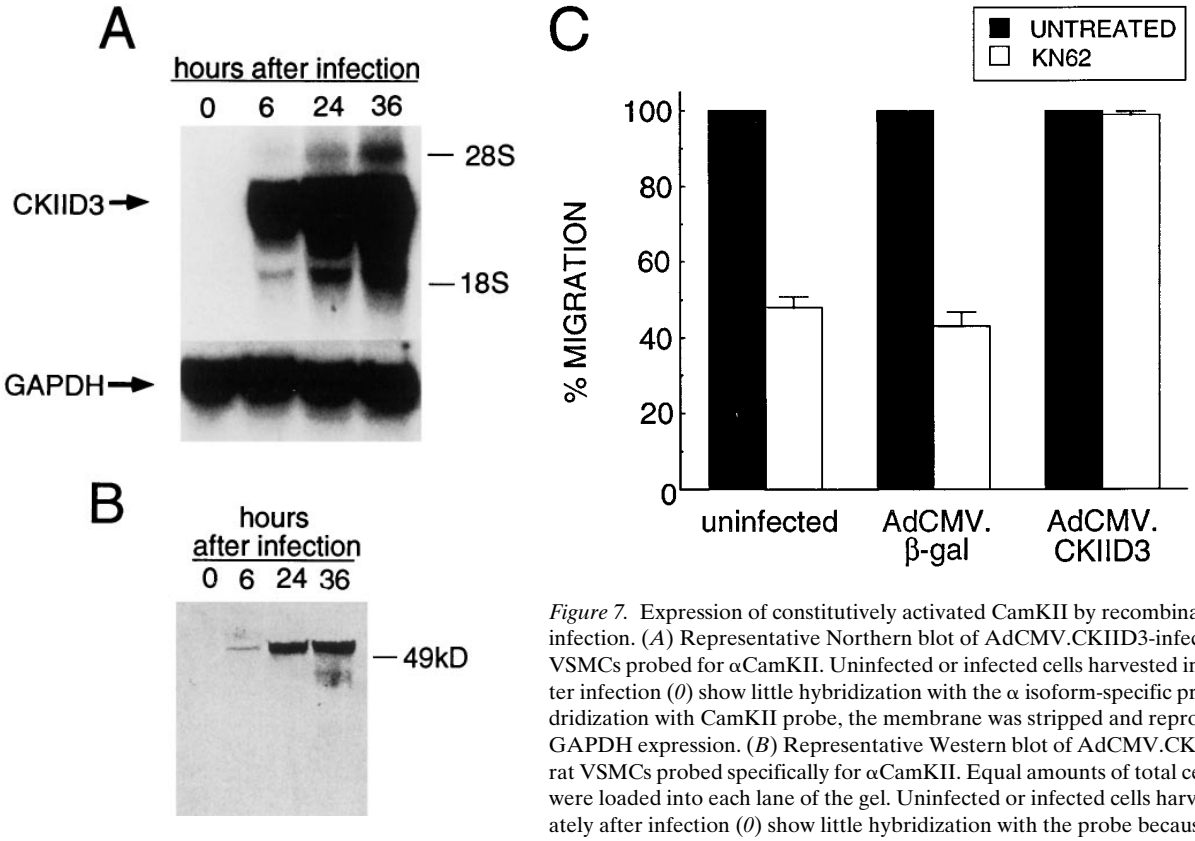

Figure 7. Expression of constitutively activated CamKII by recombinant adenoviral infection. (*A*) Representative Northern blot of AdCMV.CKIID3-infected rat VSMCs probed for αCamKII. Uninfected or infected cells harvested immediately after infection (*0*) show little hybridization with the α isoform-specific probe. After hybridization with CamKII probe, the membrane was stripped and reprobed for GAPDH expression. (*B*) Representative Western blot of AdCMV.CKIID3-infected rat VSMCs probed specifically for αCamKII. Equal amounts of total cellular protein were loaded into each lane of the gel. Uninfected or infected cells harvested immediately after infection (*0*) show little hybridization with the probe because of the fact that the predominant isoform expressed by VSMCs is δCamKII. (*C*) Functional test for constitutively activated CamKII. Rat VSMCs were infected with either AdCMV.βgal or AdCMV.CKIID3 and their migration in the presence of the inhibitor of CamKII activation, KN62, toward PDGF-BB was measured. The migration of AdCMV.CKIID3-infected VSMCs was unaffected by KN62, while that of uninfected or AdCMV.βgal-infected VSMCs was inhibited significantly.

obtained when human VSMCs were infected with the recombinant virus (data not shown).

To evaluate whether the AdCMV.CKIID3 infection of VSMCs resulted in production of a functionally active molecule, we examined the migration of infected VSMCs in presence of KN62. As shown in Fig. 7 *C*, the addition of 10 μM KN62 to the upper chamber of the Boyden apparatus significantly reduced the migration of both uninfected and AdCMV.βgal-infected VSMCs. In contrast, VSMCs infected with AdCMV.CKIID3 were completely insensitive to the effects of KN62 (percentage of migration compared to KN62-untreated cells = 99.1).

The ability of infected rat VSMCs to migrate toward PDGF in presence or absence of 1 mg/ml of cRGD peptide is shown on Fig. 8 *A*. Whereas infection with either AdCMV.-CKIID3 or AdCMV.βgal caused a small overall reduction in the number of migrating cells, more than an eightfold increase in migration occurred in response to PDGF-BB. The migration of cells infected with AdCMV.CKIID3, however, was no longer sensitive to the inhibitory effects of the cRGD peptide, whereas the migration of cells infected with AdCMV.βgal and treated with the cRGD peptide was inhibited by > 50%. The magnitude of this inhibition was similar to that seen in the absence of infection (Fig. 1).

Similar results were obtained with rat VSMC lines that stably expressed constitutively activated CamKII (D3 VSMCs) (Fig. 8 *B*). The control for these experiments were cells stably expressing the wild-type cDNA for the rat brain CamKII α-subunit (WT VSMCs). PDGF-BB was a strong chemoattractant for these cells and induced a > 10-fold increase in migration compared to BSA (D3: 87±5.4 cells per field versus 8±2.7 cells per field; WT: 88±2.2 cells per field versus 7±2.3 cells per field). D3 VSMCs, however, were completely insensitive to the effects of cRGD peptide (86±3.6 cells per field), while blocking the vitronectin receptors in WT VSMCs reduced the number of the cells that migrated toward PDGF-BB by more than 50% (43±2.9 cells per field). These experiments show that the transfected CamKII needed to be activated to block the effects of cRDG peptide and exclude the possibility that overexpression of CamKII was affecting cell migration by acting as a calmodulin sink.

Overexpression of CamKII D3 gene not only restored the ability of human VSMCs to migrate toward PDGF in presence of the cRGD peptide, but also after treatment with the $\alpha_v\beta_3$-specific monoclonal antibody LM609 (Fig. 9). While human VSMCs infected with AdCMV.βGal (*white bars*) showed an ~ 55–60% reduction in migration in the presence of the cRGD peptide (1 mg/ml) or LM609 (50 μg/ml), these same antagonists were ineffective in human VSMCs infected with AdCMV.-CKIID3.

cRGD peptides suppress PDGF-induced changes in cellular IP3 content. To determine how the cRGD peptides could suppress CamKII activation in response to PDGF-BB, we examined the effect of this antagonist on PDGF-stimulated IP3 formation. Changes in IP3 content are likely to be responsible for the initial rapid phase of intracellular calcium release in VSMCs after PDGF stimulation (38). Fig. 10 shows that in untreated cells, PDGF BB leads to an approximately twofold increase in IP3 content at 2 min after stimulation (the time at which the maximum increase occurs; Curto, K., unpublished data). Pretreatment of the cells with 1 mg/ml cRGD significantly blunted this PDGF-stimulated increase.

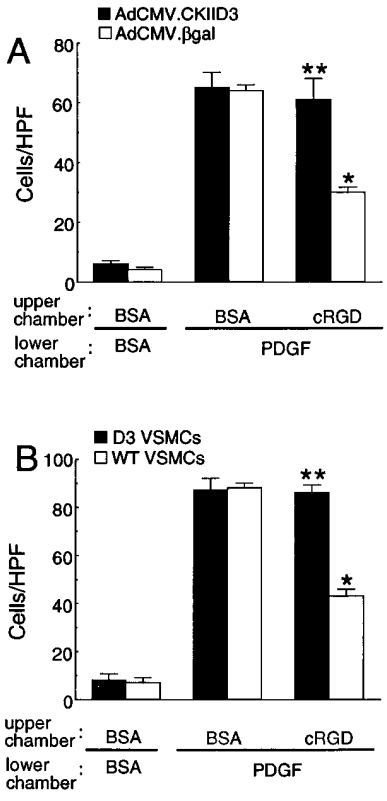

Figure 8. Expression of constitutively activated CamKII restores PDGF-directed migration to cRGD peptide-treated rat VSMCs. (*A*) PDGF-BB–directed migration of rat VSMCs infected with AdCMV.-CKIID3 or AdCMV.-βgal in the absence or presence of cRGD peptide. Absolute values for untreated and cRGD-treated Ad-CMV.CKIID3 cells: 65±5.2 versus 61±7.2 cells per HPF; for untreated and treated cRGD-treated Ad-CMV.βgal cells: 64±2.3 versus 30±2.9 cells per HPF. *Statistically significant from infected cells in the absence of cRGD, $P < 0.001$; **not statistically significant from infected cells in the absence of cRGD. (*B*) PDGF-BB–directed migration of rat VSMC lines stably expressing either wild-type (WT) or D3 mutant αCamKII (CamKIID3). The characterization of these lines have been described previously (37). *Statistically significant from WT-CamKII–expressing VSMCs in the absence of cRGD, $P < 0.001$; **not statistically significant from CamKIID3-expressing VSMCs in the absence of cRGD.

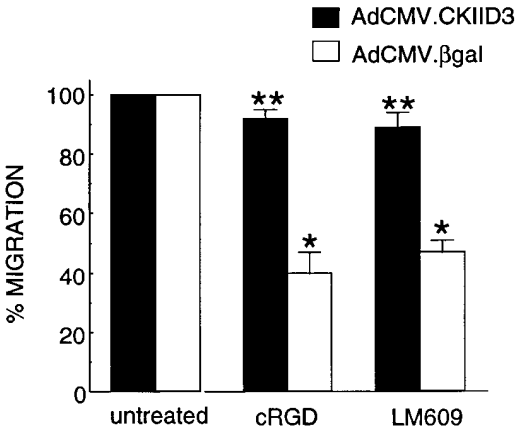

Figure 9. Expression of constitutively activated CamKII restores PDGF-directed migration to cRGD peptide and LM609-treated human VSMCs. PDGF-BB–directed migration of human VSMC infected with AdCMV.CKIID3 or AdCMV.βgal in the absence or presence of either the cRGD peptide or LM609 antibody. *Statistically significant from infected cells in the absence of cRGD, $P < 0.001$; **not statistically significant from infected cells in the absence of cRGD or LM609.

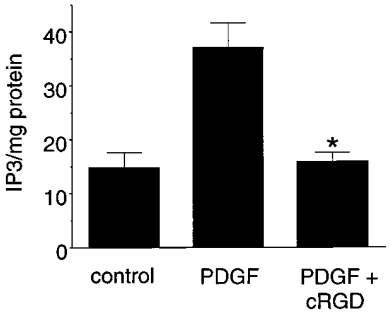

Figure 10. PDGF BB-stimulated IP3 production in rat VSMCs treated with cRGD. Total IP3 cellular content (pmol per mg of protein) in unstimulated (*control*) and PDGF-stimulated VSMCs in absence (*PDGF*) or presence of cRGD peptide (*PDGF + cRGD*). VSMCs were serum starved for 48 h and then stimulated with PDGF for 2 min (50 ng/ml). cRGD peptide (1 mg/ml) was added to cell cultures 30 min before PDGF stimulation. IP3 assay was performed as described in Methods. *Statistically significant from PDGF alone, $P < 0.001$.

In PDGF-stimulated cells, IP3 is produced from phosphatidylinositol (4,5) bisphosphate [PI(4,5)P2] through the action of PLCγ, which requires tyrosine phosphorylation after its association with the PDGF receptor. To determine whether the blunting of the IP3 response in cRGD-treated rat VSMCs was a consequence of the suppression of PLCγ activation in response to PDGF-receptor, the relative level of PLCγ tyrosine phosphorylation was measured by immunoprecipitating PLCγ from cell extracts and then Western blotting the immunoprecipitate with a phosphotyrosine-specific antibody. The results of this determination are shown in Fig. 11. The relative level of PLCγ tyrosine phosphorylation increased dramatically upon the addition of PDGF BB and was unaffected by preincubating the cells with 1 mg/ml cRGD peptide.

Discussion

Our results demonstrate that occupancy of the integrin complex, $\alpha_v\beta_3$, by either cRGD peptide, β3 integrin antibodies, or complex-specific antibodies, effectively suppresses both rat and human VSMC chemotaxis in response to PDGF-BB. This inhibition occurs without affecting overall cell attachment to the fibronectin coating used in the assay chamber. In addition, the inhibition of chemotaxis caused by the peptides and antibodies was associated with a reduction in the activation of the multifunctional protein kinase, CamKII, an enzyme that previously has been shown to play an important role in regulating VSMC migration (37, 38). Inhibition of chemotaxis after occupancy of $\alpha_v\beta_3$, was overcome through exogenous expression of a mutant CamKII cDNA that had been locked in a constitutively activated state. Migration was also restored when intracellular calcium levels were elevated independently of growth factor-mediated pathways using the calcium ionophore, ionomycin. The restoration in this case was sensitive to the specific inhibitor of CamKII, KN62, suggesting that the beneficial effects of elevating intracellular calcium were largely due to the chemoattractant-independent activation of endogenous CamKII.

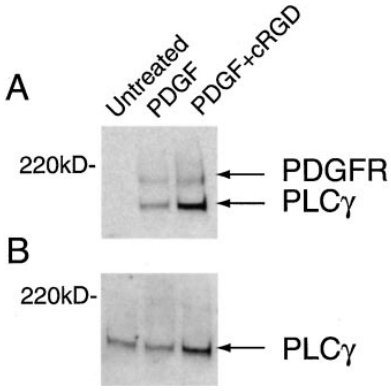

Figure 11. PLCγ activation by PDGF-BB in cRGD peptide-treated rat VSMCs. (*A*) Representative phosphotyrosine blot of rat VSMC extracts immunoprecipitated with antibodies to PLCγ and then separated by SDS-PAGE on a 6% gel. The arrows mark the position of PLCγ and the PDGFβ-receptor which were determined by reblotting the membrane with specific antibodies after stripping. (*B*) Same membrane shown in *A*, probed with antibodies to PLCγ after stripping as described in Methods.

In human VSMCs, the integrin complex responsible for modulating PDGF-directed migration was directly identified as $\alpha_v\beta_3$ through the use of the $\alpha_v\beta_3$-specific monoclonal antibody, LM609. This antibody blocked the adhesion of VSMCs to thrombospondin but not fibronectin and inhibited VSMC chemotaxis toward PDGF. A function-blocking monoclonal antibody to $\alpha_v\beta_5$ had no effect on the PDGF-directed chemotaxis of human VSMCs. For rat VSMCs, an $\alpha_v\beta_3$-specific monoclonal antibody is not available, but the fact that the inhibitory effects of either cRGD peptides, which apparently target α_v-containing integrin complexes, or β3 integrins are reversed by constitutitively activated CamKII, suggests that the responsible integrin complex in rat VSMCs is also $\alpha_v\beta_3$.

It is clear from studies in other cell types that integrins, in general, and $\alpha_v\beta_3$, in particular, not only mediate cell adhesion to various substrata, but also affect numerous cellular functions, including differentiation, gene expression, proliferation, movement, and apoptosis (53). For example, the viability and growth of human melanoma cells in three-dimensional dermal collagen requires $\alpha_v\beta_3$ receptor occupancy (28). Integrin-mediated ECM interactions necessary for cell survival have also been reported in human endothelial cells (53). Moreover, $\alpha_v\beta_3$ antagonists selectively induce apoptosis in vascular cells that have been stimulated to undergo angiogenesis (29). Recently, it has been observed that the systemic administration of monoclonal antibodies directed to $\alpha_v\beta_3$ integrin significantly reduced angiogenesis and growth of human breast carcinoma in a mouse/human chimeric model (30). Furthermore, occupancy of luminal $\alpha_v\beta_3$ in capillary endothelium modulates the permeability of the lung microvascular barrier (54) and elicits a signal leading to an enhanced invasiveness and production of type IV collagenase in human melanoma cells (27).

A critical role for $\alpha_v\beta_3$ in the pathogenesis of vascular disease is suggested by the following observations. While $\alpha_v\beta_3$ is absent in normal blood vessels, it is detectable in intimal lesions associated with various stages of atherosclerotic lesion development (31). A number of extracellular matrix molecules that interact rather selectively with $\alpha_v\beta_3$ are upregulated after vessel injury and in atherosclerotic lesions. These include thrombospondin, tenascin, and osteopontin, which are often referred to as matricellular proteins, because they interact with both cell surface receptors (including $\alpha_v\beta_3$) and other extracellular matrix proteins (50, 55). Furthermore, cyclic peptide antagonists of the broad class of vitronectin receptors effectively inhibit neointimal formation after balloon catheter injury of rabbit and hamster arteries (32, 33). Indeed, it has been suggested that the long-term beneficial effects of humanized β3

antibody on restenosis-related outcomes in humans may be related to its ability to inhibit not only platelet activation and aggregation through the $\alpha_{IIb}\beta_3$ receptors, but also later vascular events mediated by $\alpha_v\beta_3$ (34). Previous in vitro studies with isolated vascular smooth muscle cells have demonstrated that at least one of these effects acts on PDGF-directed migration, suggesting that occupancy of vitronectin receptors, such as $\alpha_v\beta_3$, modulates intracellular signaling initiated by soluble chemoattractants, such as PDGF-BB (32). The observations reported here suggest a general mechanism for integrating ECM-dependent and chemoattractant-mediated intracellular signaling pathways involved in the regulation of VSMC migration.

This mechanism highlights a critical regulatory role for the multifunctional protein kinase, CamKII, in chemotaxis. Previous studies have established that CamKII activation is crucial in other situations in which the PDGF-directed migration of VSMCs is blocked. These include growth arrest (37) and the application of antibodies or antisense oligonucleotides that either neutralize or inhibit endogenous bFGF expression (38). In both cases, there is also a significant or complete reduction in CamKII activation after the administration of PDGF-BB. Furthermore, chemotaxis in both circumstances is restored completely through either forced elevation of intracellular calcium (which causes PDGF-BB-independent activation of CamKII) or forced exogenous expression of a constitutively activated mutant CamKII cDNA. The blockade of PDGF-directed chemotaxis by neutralization of endogenously produced bFGF could also be reversed if exogenous bFGF was supplied during the migration. We show here that exogenous bFGF also rescues VSMCs from the inhibitory effects of either the cRGD peptides or β3-integrin antibodies and that this rescue is also dependent on CamKII activation (Fig. 6). These results indicate that the requirement for bFGF is downstream of $\alpha_v\beta_3$ integrin regulation and suggest that occupancy of the $\alpha_v\beta_3$ regulates bFGF production/secretion.

How integrin occupancy influences the activation of CamKII is unknown. It is thought that the activation of CamKII is the result of the rapid release of calcium from intracellular stores triggered by an elevation in IP3 content (56, 57). Our data show that pretreatment of VSMCs with the cRGD peptide blocks the increase in IP3 content that normally occurs in response to PDGF. IP3 is generated along with diacylglycerol (DAG) by the enzyme, PLCγ (58), which in response to PDGF associates with the PDGF receptor and becomes tyrosine phosphorylated. Phosphorylation of PLCγ at specific tyrosines has been shown to be necessary for enzyme activity (59). As we show here, however, tyrosine phosphorylation of PLCγ in VSMCs in response to PDGF is unaffected by the cRGD peptides (Fig. 10). It is possible that, while tyrosine phosphorylation of PLCγ is required, it is not sufficient for full in vivo activity. Indeed, evidence suggests that phosphorylation of PLCγ on serine residues may negatively modulate its activity (60). IP3 formation, however, could also be affected by substrate availability. For example, while intracellular calcium is rapidly mobilized in PDGF-stimulated fibroblast adhering to tissue culture plastic or immobilized ECM, the stimulation of suspended fibroblasts fails to do so (61, 62). In fact, IP3 formation in response to PDGF is suppressed in suspended cells because of a reduction in the substrate PI(4,5)P2 pool (63). The activity of the enzyme responsible for PI(4,5)P2 formation, namely phosphatidylinositol phosphate (PIP) 5-kinase (PI5-K), is, in turn, regulated by integrin activation through a mechanism involving the small GTP-binding protein, rho, and is suppressed in suspended fibroblasts (64). Unlike cells in suspension, however, the VSMCs used in this study that were treated with the cRGD peptide and integrin antibodies remained attached to the ECM, suggesting that the majority of integrin-associated focal contacts with the ECM persist in the presence of the cRGD peptides and β3 or $\alpha_v\beta_3$ antagonists. Therefore, unless PIP 5-K specifically associates with vitronectin receptors in these cells, its activity and the level of PI(4,5)P2 would be expected to remain unchanged.

In our experimental system, migration occurs toward a PDGF gradient that is established across a filter coated with fibronectin. Neither the antibody to $\alpha_v\beta_3$ nor the cRGD peptide interfered significantly with attachment to this substrate, raising the question as to the identity of the extracellular matrix component(s) interacting with $\alpha_v\beta_3$ to regulate migration. It is possible that total $\alpha_v\beta_3$ levels are so low relative to that of the other cell surface binding sites for matrix proteins that their contribution to overall adhesion is minimal. On the other hand, $\alpha_v\beta_3$ may interact with endogenously produced ECM proteins. Giachelli et al. (55), for example, have reported that several growth factors, including PDGF-BB, increase the mRNA levels and protein expression of the matricellular protein, osteopontin, which, in turn, interacts with $\alpha_v\beta_3$ to drive osteopontin-directed cell migration (50). Other extracellular matrix molecules produced by VSMCs that may interact with $\alpha_v\beta_3$ include TSP and tenascin. TSP is unique among other ECM binding proteins in that it not only binds to $\alpha_v\beta_3$ but also to the 50-kD integrin-associated protein (IAP) through a separate domain on the molecule (51). Because IAP tightly associates with β3 integrins, it is possible that a single TSP molecule could engage both IAP and $\alpha_v\beta_3$. Such dual engagement may be necessary for signaling from the complex. As is the case for osteopontin, TSP expression is also upregulated by PDGF-BB in cell cultures and by injury to the vessel wall in vivo (65).

Our observations on the regulation of VSMC chemotaxis by integrin occupancy share many similarities to those reported by Blystone and colleagues (66, 67) for K562 cells transfected with cDNAs for both α_v and β_3 integrins. These investigators found that ligation of the resulting $\alpha_v\beta_3$ complex with either antibody or soluble vitronectin inhibited $\alpha_5\beta_1$-mediated phagocytosis, a process involving recognition of fibronectin coated particles, but had no effect on $\alpha_5\beta_1$-mediated adhesion to fibronectin. We have shown here that antibodies to $\alpha_v\beta_3$ inhibit chemotaxis of VSMCs to PDGF on a fibronectin substrate without affecting VSMC adhesion to fibronectin. In the $\alpha_v\beta_3$-transfected K562 cells, the inhibition of $\alpha_5\beta_1$ phagocytosis by $\alpha_v\beta_3$ was reversed by inhibiting a serine/threonine kinase, with an inhibitor profile consistent with, but not exclusive for, protein kinase C. On the other hand, we have shown that $\alpha_v\beta_3$ suppression of migration is accompanied by the failure of PDGF to stimulate another serine/threonine kinase, namely CamKII and that the effect of $\alpha_v\beta_3$ can be reversed by expression of a constitutively activated CamKII. The relationship between the serine/threonine kinase which negatively regulates phagocytosis in K562 cells and CamKII, whose activity is required for VSMC migration, remains to be determined.

The K562 experiments and other data have lead to the notion that ligand binding to one integrin can affect the functions of other integrins, a process termed "integrin crosstalk" (66,

67). In the case of the K562 cells, it is speculated that $\alpha_v\beta_3$ affects the affinity state of $\alpha_5\beta_1$ so that low affinity functions such as adhesion are not affected, but higher affinity function, such as phagocytosis, are inhibited. It is possible that a similar phenomenon is occurring in anti-$\alpha_v\beta_3$ treated VSMCs, namely that the ligated $\alpha_v\beta_3$ integrin complex may affect the affinity state of other integrins. Indeed, recent experiments indicate that a decrease in the activation state of β1 integrins is seen in vivo after balloon catheter injury to vessels (68). Likewise, the treatment of human VSMCs with an antibody that induces the activated or high affinity state for β1 integrins suppresses the migration toward PDGF in cell culture (69).

The results presented here provide the outline of a mechanism by which traditional growth factor–signaling pathways are regulated through interactions of the cell with the ECM. In response to vessel injury, a gradient of PDGF is invariably established across the vessel wall, originating either from activated platelets that adhere to the denuded endothelium or macrophages that are recruited to sites of developing atherosclerotic lesions (1). The work of others has shown that the expression of both $\alpha_v\beta_3$ integrin and thrombospondin are also upregulated in balloon-injured vessels and atherosclerotic lesions (31, 55, 66). According to our data, outside–in signaling from the occupied $\alpha_v\beta_3$ integrin complex then enables PDGF to activate CamKII and for cell migration to proceed. The signaling events downstream of CamKII activation that are important for cell movement are currently unknown and are the subject of continuing investigation. The results reported here provide the first demonstration of how ECM interactions with VSMCs regulate intracellular signaling pathways and possibly contribute to the pathogenesis of vascular lesions.

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