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Regulation of Vascular Smooth Muscle Migration by Mitogen-activated Protein Kinase and Calcium/Calmodulin-dependent Protein Kinase II Signaling Pathways

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M. S. LUNDBERG, K. A. CURTO, C. BILATO, R. E. MONTICONE AND M. T. CROW. Regulation of Vascular Smooth Muscle Migration by Mitogen-activated Protein Kinase and Calcium/Calmodulin-dependent Protein Kinase II Signaling Pathways. *Journal of Molecular and Cellular Cardiology* (1998) 30, 2377–2389. Platelet-derived growth factor BB (PDGF BB) activation of the mitogen-activated protein kinases (MAPK), ERK1 and ERK2, has been shown to be necessary for mitogen-stimulated proliferation, but its role in regulating cell migration and its relationship to other chemotactic signaling events, such as CamKII activation, has not been defined. Using a modified Boyden chamber apparatus, we tested the effects of a selective inhibitor of the upstream activator of ERK1/2, MEK1, on PDGF-stimulated rat aortic vascular smooth muscle cells (VSMCs) alone and in combination with KN62, a selective inhibitor of CamKII. The MEK1 inhibitor, PD98059, caused a dose-dependent reduction in ERK2 activity that paralleled a decrease in migration up to 60%. This inhibition of migration was similar to that seen with KN62 and the combined effects of both inhibitors were non-additive. Although KN62 did not affect ERK2 activity in response to PDGF, PD98059 markedly inhibited PDGF-stimulated CamKII activity, suggesting that activation of CamKII by PDGF was dependent on ERK activity and that the effects of ERK inhibition on migration may be mediated through its ability to inhibit CamKII activity. To directly test this, VSMCs were infected with a recombinant adenovirus expressing constitutively activated CamKII. Infection reversed the inhibitory effects of KN62 on migration, but had no effect on the inhibition of migration seen with PD98059. These results suggest that while MAPK may act upstream of CamKII to control its activation in response to PDGF, it also regulates migration independently of CamKII activation. © 1998 Academic Press

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Introduction

Platelet-derived growth factor (PDGF) is an important factor mediating the proliferative and migratory responses of vascular smooth muscle

cells (VSMCs) associated with various vascular diseases and disorders (Ross, 1993). The importance of PDGF in the intact vessel is highlighted by studies which show that antibody neutralization of PDGF markedly reduces the vessel wall's response

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to mechanical injury (Ferns *et al.*, 1991; Jawien *et al.*, 1992). *In vitro*, PDGF BB has been shown to stimulate phosphatidylinositol turnover which, in turn, may be linked with cytoskeletal actin reorganization and cell migration (Stossel, 1993; Bornfeldt *et al.*, 1995). Previous studies from our laboratory demonstrated that PDGF BB-directed migration of VSMCs is largely mediated by and regulated through activation of the multifunctional protein kinase, calcium/calmodulin-dependent protein kinase II (CamKII) (Pauly *et al.*, 1995; Bilato *et al.*, 1995, 1997). PDGF-stimulated CamKII activity results from increased cytosolic calcium levels due to phospholipase C γ activation and subsequent increases in phosphoinositide metabolism. PDGF BB also potently activates the MAPK cascade, a well-characterized pathway necessary for cell proliferation and signaling in response to stress (Pazin and Williams, 1992; Seger and Krebs, 1995). One MAPK pathway activates extracellular signal-regulated kinases 1 and 2 (ERK1/2), a proline-directed serine/threonine protein kinase whose activity is stimulated by diverse external stimuli. ERK1/2 are activated by dual phosphorylation on both tyrosine and threonine residues, a process carried out by the dual-specificity protein kinase, MAPK/*erk* kinase (MEK) (Nakielnny *et al.*, 1992). Phosphorylation of ERK1/2, in turn, leads to phosphorylation of many different regulatory proteins localized in various cellular compartments, such as the nucleus, cytosol, membrane, and cytoskeleton (Boulton *et al.*, 1991; Pulverer *et al.*, 1991; Seger and Krebs, 1995).

Although VSMC migration from the media to the intima is thought to be a key event in the pathogenesis of atherosclerosis and restenosis (Bilato and Crow, 1996), the signaling pathways controlling VSMC migration are not well defined. The aim of the present study was to identify whether activation of ERK1/2 is necessary for VSMC chemotaxis toward PDGF BB. We have examined the effects on PDGF-directed migration of inhibiting this pathway with the selective MEK inhibitor, PD98059 (Alessi *et al.*, 1995), comparing the effects of the inhibitor alone and in combination with KN62, a selective inhibitor of CamKII (Tokumitsu *et al.*, 1990). We show that both PD98059 and KN62 decrease PDGF-directed VSMC migration but that the combined effects of the two inhibitors were no greater than the maximum effect of either one alone. PD98059 also selectively inhibited CamKII activation in response to PDGF. Because our previous studies demonstrated that activation of CamKII is a critical regulatory event in VSMC

migration (Pauly *et al.*, 1995; Bilato *et al.*, 1995, 1997), we tested the hypothesis that the ERK signaling pathway's effect on migration was mediated through its effects on CamKII activation. VSMCs were infected with a recombinant adenovirus expressing constitutively-activated CamKII and then treated with either PD98059 or KN62 alone or in combination. As expected, constitutively-activated CamKII fully restored migration to KN62-treated cells, but failed to alter the inhibition caused by PD98059 alone or together with KN62. These results demonstrate that the ERK signaling pathway regulates VSMC migration through both CamKII-dependent and independent mechanisms.

Materials and Methods

Materials

Recombinant human PDGF BB (Collaborative Research, Lexington, MA) was dissolved in either Dulbecco's modified Eagle's Medium (DMEM) containing 0.1% BSA and stored at -70°C for migration assays, or in 10 mM acetic acid containing 1mg/ml bovine serum albumin (BSA) for MAP kinase assays. Calmodulin (Upstate Biotechnology, Inc., Lake Placid, NY) and autocalmitide-2 (Peninsula Laboratories, Inc., Belmont, CA) were dissolved in water. Myelin basic protein (MBP) was obtained from Gibco/BRL (Gaithersburg, MD) and dissolved in assay buffer (20 mM MOPS, 2 mM EGTA, 10 mM MgCl_2 , 0.1% Triton X-100, 1 mM dithiothreitol [DTT]). PD98059 was purchased from New England Biolabs (Beverly, MA) and reconstituted in DMSO. 1-[N-(0-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl-4-phenylpiperazine (KN62) was purchased from Alexis Laboratories (San Diego, CA) and dissolved in DMSO. GPenGRGDSPCA (cRGD) was obtained from Gibco/BRL (Gaithersburg, MD), dissolved in water, and stored at -4°C . All other aliquoted reagents were stored at -20°C and used only once after thawing. Unless otherwise stated, cells were pretreated with inhibitors 30 min prior to the start of the assay.

Cell culture

Enzymatically dissociated aortic medial VSMCs were established in culture from 3- to 6 month-old Wistar rats as described previously (Pauly *et al.*, 1994, 1995, 1998). Proliferating cells were routinely maintained in DMEM/high glucose (Gibco/BRL Cat

11995-065, Gaithersburg, MD) supplemented with 10% fetal calf serum, 1 mM non-essential amino acids, 50 $\mu\text{g}/\text{ml}$ penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin and 100 $\mu\text{g}/\text{ml}$ neomycin (GibcoBRL) in a humidified 5% CO_2 atmosphere at 37°C. Cells between the 5th and 10th passage were used for all experiments. Cultures were serum-starved overnight before use in migration experiments or biochemical analyses.

VSMC migration assay

Migration assays were performed using a modified Boyden chamber as previously described (Pauly *et al.*, 1994, 1998). Briefly, polycarbonate filters (13 mm diameter, 8 μm pores, Nucleopore Corp., Pleasanton, CA) were coated either with a mixture of either 50 $\mu\text{g}/\text{ml}$ purified native collagen type I (Upstate Biotechnology Inc., Lake Placid, NY) and fibronectin (Gibco/BRL, Gaithersburg, MD), or 50 $\mu\text{g}/\text{ml}$ fibronectin alone, or 50 $\mu\text{g}/\text{ml}$ gelatin (Sigma Chemical Co, St. Louis, MO), all of which were dissolved and diluted in 10 mM acetic acid. Coated filters were allowed to air-dry overnight in a sterile environment before use. Boyden chambers were assembled by adding 10 ng/ml PDGF BB in DMEM to the lower (chemoattractant) chamber. Cells were pretreated for 30–60 min with inhibitors used at the concentrations indicated in the results and then resuspended in DMEM containing 0.1% BSA and added to the upper chamber in a final volume of 0.8 ml. The chambers were then incubated for 4 h at 37°C in a 5% CO_2 humidified environment. At the end of the incubation period, cells migrating through to the lower side of the filter were fixed, stained and counted as described previously (Pauly *et al.*, 1998).

For experiments using gelatin-coated filters, we found it necessary to first pre-attach VSMCs to the filters, transfer the filter to a new chamber set-up, and then pretreat with PD98059 after attachment had occurred, since the adhesion data reported in Figure 3 show that pretreatment with PD98059 selectively blocked VSMC attachment to gelatin-coated surfaces.

Cell adhesion assay

The individual wells of a 96-well microtiter plate (Nunc Maxisorp, Naperville, IL) were coated overnight at 4°C with a mixture of either 50 $\mu\text{g}/\text{ml}$ purified native collagen type I and fibronectin, or 50 $\mu\text{g}/\text{ml}$ fibronectin alone, or 50 $\mu\text{g}/\text{ml}$ gelatin.

Non-specific binding sites were blocked with 5 mg/ml heat-denatured BSA for 1 h at 37°C. Adhesion to the substrate for 1 h at 37°C was measured after adding 30 000 cells/well resuspended in DMEM and 1% BSA. The extent of adhesion was measured after gently rinsing away non-adherent cells with PBS. Remaining cells were fixed in 4% formaldehyde in PBS. The microplate was then stained with 0.5% toluidine blue in 4% formaldehyde for 5 min. Each well was then rinsed extensively with water and the stain was eluted from the adherent cells with 1% SDS for 15 min at room temperature. Absorbance from each well was measured at 595 nm using a microplate reader (BioRad model no. 3550, Pleasanton, CA).

Recombinant adenovirus vector infection of VSMCs

Characterization of a replication-defective adenovirus expressing a constitutively-activated CamKII (AdCMV.CKIID3) in which amino acids 286 and 287 were converted to aspartic acid has been previously described in detail (Bilato *et al.*, 1997). An adenovirus containing no cDNA insert (AdCMV.null) or one expressing the *E. coli* β -galactosidase gene (AdCMV. β gal) were used as infection controls. VSMCs at 30–50% confluence bathed in serum-free media at approximately 0.04 ml/cm² (3 ml/100 mm plate) were infected at a multiplicity of infection (m.o.i.) of 100 for 1 h with occasional rocking of the culture plate. Additional media containing 10% FCS was then added to bring the culture volume up to 0.15–0.2 ml/cm². Virus-containing medium was replaced after 24 h and the cultures were used for migration assays and biochemical analyses within 48 h of infection.

CamKII activity assay

Activity of CamKII in response to PDGF BB and other reagents was measured in cell extracts using a synthetic peptide substrate as previously described (Pauly *et al.*, 1995; Bilato *et al.*, 1995, 1997). Total CamKII was determined in a reaction volume containing calcium and calmodulin, whereas autonomous activity was measured in another aliquot of the sample using the same reaction mixture without calcium or calmodulin. Values are expressed as the per cent activity, that is, calcium/calmodulin independent (autonomous activity) relative to total activity.

ERK2 activity assay and ERK1/2 phosphorylation

Cells were plated in 100 mm dishes and starved in DMEM supplemented with 0.1% FBS for 20–24 h before treatment. After treatment, they were rinsed twice in ice-cold PBS and lysed by scraping at 4°C in ERK Buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β -glycerolphosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, 10 U/ml aprotinin, 5 mM sodium fluoride, 1 mM DTT). After centrifugation to produce a cleared lysate, total protein content was quantified using the BCA determination method. Fifty to one hundred micrograms of lysate proteins were immunoprecipitated overnight with ERK2 antibody (1:1000 dilution SC154; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 50 μ l of a 50% slurry of Protein A-Sepharose beads (Sigma Chem. Co, St. Louis, MO). The pellets were then washed three times with ERK Buffer, three times with buffer 2 (0.5 M LiCl, 100 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM DTT) and three times with assay buffer (listed under "Materials"). The immune complexes were re-suspended in assay buffer containing 50 μ M ATP, 15 μ Ci of [γ - 32 P]ATP (specific activity > 1000 Ci/mmol) and 0.5 mg/ml MBP as substrate. Kinase activity was assayed for 20 min at 30°C and the reaction was stopped by addition of 5X Laemmli's sample buffer. Proteins were resolved by 15% SDS-gel electrophoresis, fixed in 10% acetic acid, dried under vacuum and subjected to autoradiography. Incorporation of 32 P was analysed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

The relative levels of activated ERK1/2 protein was measured using a dual phosphospecific antibody (Promega Corp, Madison, WI). After serum-starvation and treatment, a cell extract was prepared by scraping in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 mM PMSF) and then clarified by centrifugation (16 000 \times g) for 10 min. The supernatant was removed, transferred to a fresh tube, sonicated for 20 s, and total protein content determined (BCA reagents, Pierce, Rockford, IL). Extracts were mixed with 3X sample buffer (188 mM Tris-HCl, pH 6.8, 6% w/v SDS, 30% glycerol, 0.15 M DTT, 0.1% bromophenol blue), boiled for 3 min and quenched on ice. Eight micrograms of protein was loaded in each lane, separated by 10% SDS-PAGE (Novex, San Diego, CA) and electrotransferred to polyvinylidene-fluoride membranes (Millipore Corp, Bedford, MA)

in a semi-dry apparatus (Bio-Rad Laboratories, Richmond, CA) at 5 V overnight. Membranes were blocked in 1% BSA in PBS/0.1% Tween-20 for 1 h at room temperature. The membrane was then probed for 1 h at room temperature with an antibody recognizing activated ERK1/2 diluted 1:10 000 in Western Buffer (50 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.1% Tween-20). Antigen-antibody complexes were visualized by chemiluminescence (Pierce, Rockford, IL) after incubating for 1 h at room temperature with anti-rabbit IgG conjugated to horseradish peroxidase according to the manufacturer's instructions (Amersham Corp., IL). The relative intensities of the luminescent signals captured on film were quantified by densitometry (Molecular Dynamics, Sunnyvale, CA).

Statistical methods

All data are expressed as the mean \pm s.e. The comparison of the mean values among the different groups was made by ANOVA with *P* values corrected by the Bonferroni method (Devore, 1982).

Results

PD98059 inhibits PDGF-stimulated VSMC MAPK activity and PDGF-directed VSMC migration

Figure 1 (A) shows that pretreatment with the MEK inhibitor, PD98059, markedly suppressed migration ($57 \pm 4.3\%$) toward PDGF BB in a dose-dependent manner that correlated well with its ability to block ERK2 activity (Figure 2). The half maximal inhibitory dose of PD98059 for both migration and ERK2 activity was approximately 3 μ M with the maximal inhibitory effect achieved between 25–50 μ M. PD98059 did not affect the ability of VSMCs to adhere to fibronectin that was used to coat the upperside of the filter in the Boyden apparatus [Figure 1(B)].

The magnitude of the effect of PD98059 on PDGF-directed migration of VSMCs was dependent on the type of extracellular matrix used in the Boyden apparatus. While migration was inhibited by $57 \pm 4.3\%$ for cells plated onto fibronectin, inhibition was significantly greater although somewhat variable ($78 \pm 12\%$) for cells plated on gelatin [Figure 3(A)]. Pretreatment with PD98059, however, dramatically inhibited the initial attachment of VSMCs to gelatin [Figure 3(B)] so that the migration

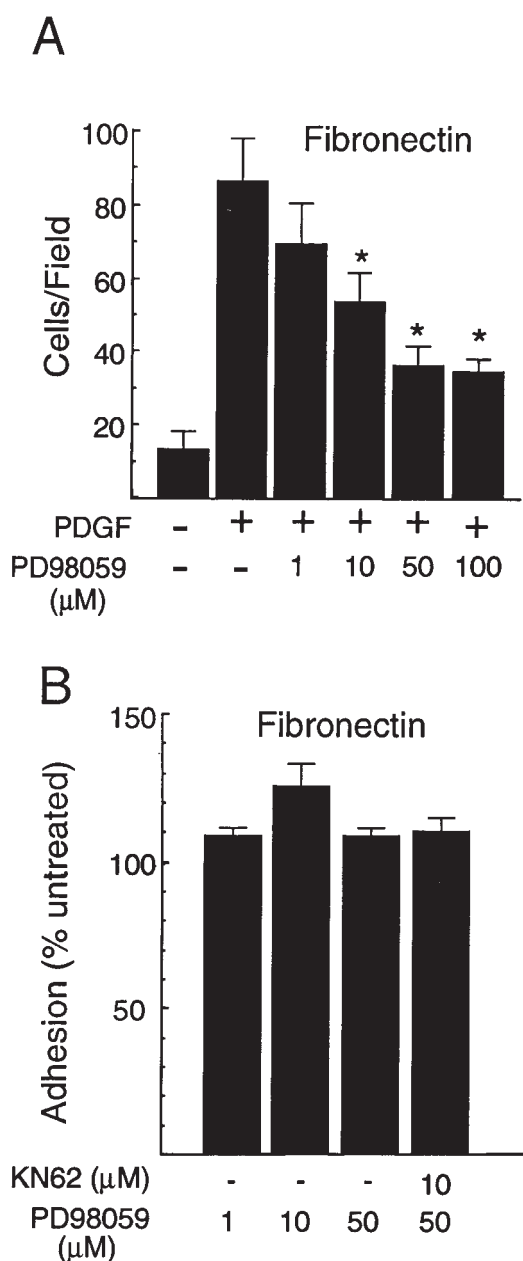


Figure 1 Inhibition of PDGF-directed migration of VSMCs plated on fibronectin by PD98059. (A) Dose response of the effect of PD98059 on PDGF-directed migration of rat VSMCs plated on fibronectin. VSMCs were serum-starved overnight and pretreated the next day for 30 min with different amounts of PD98059, and then assayed for PDGF-directed migration in the presence of PD98059 in a Boyden chamber in which the filters were coated with 50 $\mu\text{g}/\text{ml}$ rat fibronectin. *Significantly different from untreated PDGF-stimulated VSMCs ($p > 0.001$). (B) Adhesion of serum-starved VSMCs to 50 $\mu\text{g}/\text{ml}$ fibronectin in the presence of increasing amounts of PD98059, or the combination of PD98059 and KN62 following a 30-min pretreatment with the inhibitors. Data are expressed as per cent adhesion relative to untreated controls. Adhesion was unaffected by either inhibitor, whether it was applied alone or in combination.

experiments shown in Figure 3(A) were performed with the inhibitor added after attachment of the VSMCs to the gelatin-coated filter, indicated in Figure 3 by the suffix "p". Because of the variable response of VSMCs plated on gelatin to PD98059 and the additional procedural complications associated with adding PDGF and inhibitors after cell attachment, we elected to proceed in the remaining experiments with cells plated on fibronectin.

The combined effects of CamKII and ERK2 inhibition on VSMC migration

We have previously demonstrated an important regulatory role for activation of CamKII in PDGF-directed migration of VSMCs (Pauly *et al.*, 1995; Bilato *et al.*, 1995, 1997). To examine the interaction between ERK and CamKII signaling pathways with respect to directed cell movement, the combined effects of PD98059 and KN62, a specific inhibitor of CamKII, were examined. The data in Figure 4 show that pretreatment with PD98059 alone reduced migration over the 4-h assay period to $47 \pm 7\%$ of control, and that pretreatment with KN62 alone reduced migration to a comparable extent ($41 \pm 7\%$ of control). The pretreatment of VSMCs with both inhibitors, however, caused no further reduction in migration ($41 \pm 8\%$). Neither inhibitor alone or in combination affected adhesion of the VSMCs to fibronectin [Figure 1(B)] or a mixture of collagen and fibronectin (data not shown).

PD98059 inhibits CamKII activation in PDGF-stimulated VSMCs

One interpretation for the lack of an additive effect of the inhibitors is that the two signaling pathways are serially linked, with CamKII either upstream or downstream of ERK activation. To directly test this possibility, we examined the effect of each specific inhibitor on activation of the other kinase pathway. Figure 5 shows that, as expected, KN62 (10 μM) completely blocked CamKII activity in response to PDGF stimulation. Surprisingly, PDGF-stimulated CamKII activity was also blocked by the MEK inhibitor, PD98059. Figure 5 shows that CamKII activity in unstimulated cells was $13.3 \pm 1.4\%$. Addition of PDGF BB resulted in an increase in activity to $70.1 \pm 2.8\%$ which was effectively inhibited by pretreatment with either KN62 ($17 \pm 3.7\%$) or PD98059 ($21 \pm 4.2\%$). This effect of PD98059 pretreatment on CamKII activity is unlikely to be a

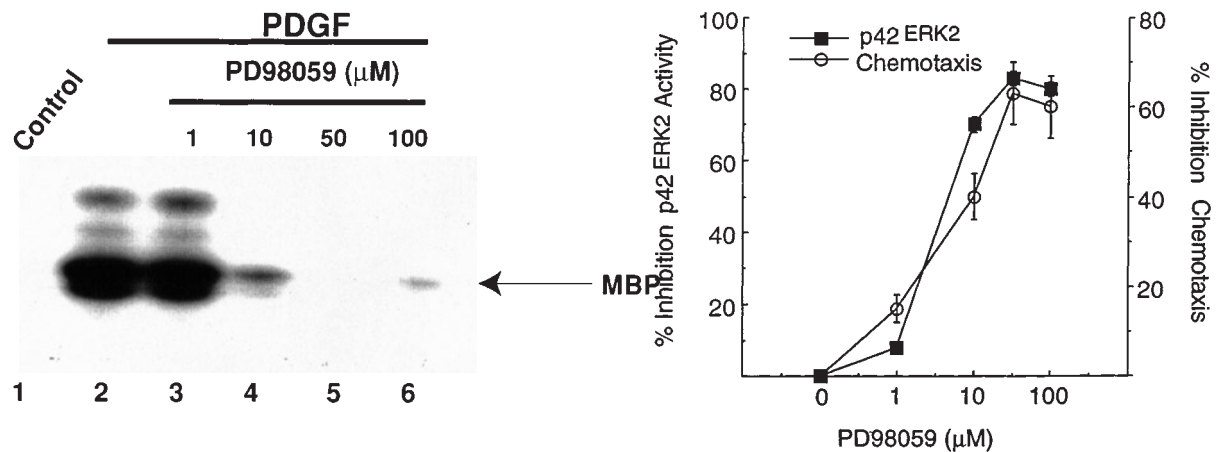


Figure 2 Inhibition of PDGF BB-induced MAPK activity by PD98059 in a dose-dependent manner. (A) Rat VSMCs were serum-starved overnight and then preincubated the next day with either vehicle alone or with the indicated concentrations of PD98059 for 30 min. Cells were then stimulated with 50 ng/ml PDGF BB for 10 min. Fifty micrograms of cell lysate were prepared and subjected to immunoprecipitation using a specific antibody to the MAPK isoform, p42^{ERK2}. The immunocomplexes were washed and phosphotransferase activity of p42^{ERK2} was assayed as the incorporation of [γ -³²P]ATP into myelin basic protein (MBP). (B) Comparison of the dose-response for inhibition of PDGF-stimulated p42^{ERK2} enzymatic activity and chemotaxis. The graph shows the dose-response for p42^{ERK2} inhibition (■) obtained by quantitative densitometry of at least three experiments similar to that shown in (A) compared to the dose-response for inhibition of chemotaxis (○) shown in Figure 1.

direct effect on the enzyme, since it was not observed in samples where the enzyme had been activated with the calcium ionophore, ionomycin (Figure 5). Because inhibition was unique to the mode of stimulation (PDGF *v* ionomycin) and did not affect basal levels of enzyme activity, this suggested that activation of ERK1/2 was required for PDGF-stimulated CamKII activity.

Effect of constitutively activated CamKII expression on PD98059-inhibited migration

If regulation of CamKII activation by MEK/ERK activation was the only mechanism by which PD98059 inhibited PDGF-directed migration, then expression of a constitutively activated CamKII should restore migration to PD98059-treated cells, which we have previously shown to be the case for migration inhibited by KN62 (Pauly *et al.*, 1995; Bilato *et al.*, 1995, 1997). To directly test this, VSMCs were infected with a recombinant adenovirus expressing a mutant CamKII "locked" into an activated state (Bilato *et al.*, 1997). Infection with a recombinant adenovirus expressing either β -galactosidase or no cDNA was used to control for the effect of infection. Infection did not alter the ability of VSMCs to migrate toward PDGF BB. Migration of uninfected VSMCs was 98 ± 7.2 cells/field in the presence of PDGF compared to 15.7 ± 6.1 cells/field in its absence. PDGF-directed migration in

AdCMV. β gal-infected and AdCMV.CKIID3-infected VSMCs was 102 ± 14 and 87 ± 11 cells/field against a background of 23 ± 8 and 17.5 ± 5 cells/field, respectively (Figure 6). Expression of constitutively-activated CamKII completely reversed KN62 inhibition of migration ($92 \pm 7\%$ of untreated cells), but had no effect on inhibition seen with either PD98059 alone ($64 \pm 9\%$) or in combination with KN62 ($57 \pm 2\%$). Infection did not result in altered PDGF-stimulated MAPK activity in the presence of either inhibitor (Figure 7).

Differential regulation of PDGF-stimulated CamKII and ERK2 activity in response to integrin blockade of VSMCs

We have recently shown that CamKII activity in VSMCs requires occupancy of the integrin $\alpha v \beta 3$ (one of two vitronectin receptors expressed on VSMCs) and that antibodies or peptides that block that occupancy inhibit both PDGF-stimulated CamKII activation and migration (Bilato *et al.*, 1997). Signaling from occupied $\alpha v \beta 3$ integrins on the surface of VSMCs represents a potentially important physiological form of regulation for these cells (Choi *et al.*, 1994; Matumo *et al.*, 1994; Hoshiga *et al.*, 1995). To determine if $\alpha v \beta 3$ integrin occupancy also affected the ability of PDGF to stimulate ERK2 activity, VSMCs were pretreated with a specific vitronectin receptor antagonist, cRGD (GPenRGDSPCA), stimulated with PDGF, and

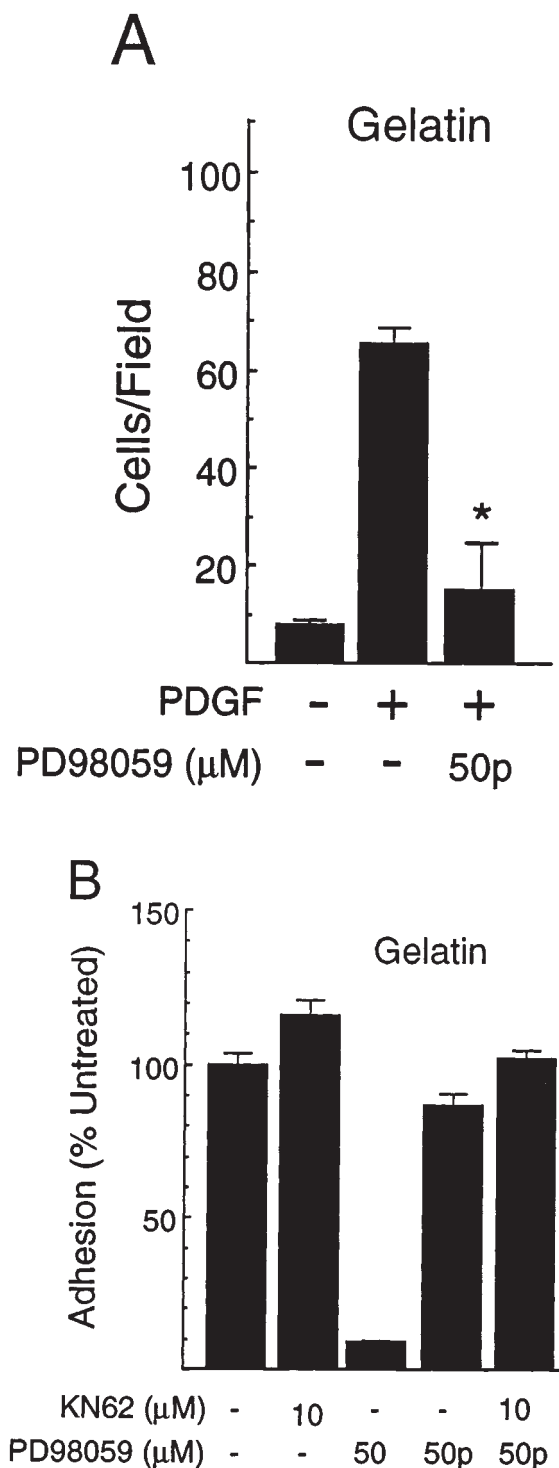


Figure 3 Inhibition of PDGF-directed migration of VSMCs plated on gelatin by PD98059. (A) Effect of 50 μM PD98059 on PDGF-directed migration of rat VSMCs plated on gelatin. VSMCs were preattached to 50 $\mu\text{g}/\text{ml}$ gelatin-coated filters in the absence of PDGF, or 50 μM PD98059 (50p) and then transferred to a Boyden apparatus in which PDGF was placed in the bottom and PD98059 in the upper chambers and the migration assay immediately started. *Significantly different from untreated PDGF-stimulated VSMCs ($p > 0.001$). (B) Ad-

then analysed for CamKII and ERK1/2 activity. As shown previously, cRGD did not affect adhesion of VSMCs to the extracellular matrix but did cause a large reduction in CamKII activity in PDGF- but not ionomycin-stimulated VSMCs [Bilato *et al.*, 1997; Figure 8(A)]. In contrast, this integrin antagonist had no effect on the ability of PDGF to stimulate ERK activation in VSMCs [Figures 8(B) and 8(C)].

Discussion

We have shown here that inhibition of the mitogen-activated protein kinase ERK2 by PD98059 is closely correlated with the inhibition of PDGF-directed VSMC migration (Figure 2). The extent of the inhibition of VSMC PDGF-directed migration by PD98059 was similar to that previously reported by the CamKII specific inhibitor KN62 and the combined effect of both inhibitors was non-additive. Interestingly, while KN62 had no effect on PDGF-stimulated ERK2 activity, PD98059 markedly inhibited CamKII activity, demonstrating that PDGF-stimulated CamKII activation required ERK2 activity. This, in turn, suggested that the effects of ERK2 inhibition on migration may be mediated by its effects on CamKII, a hypothesis consistent with the non-additive effects of combining the inhibitors. None the less, while forced expression of constitutively-activated CamKII was sufficient to reverse the inhibitory effects of KN62, it was unable to reverse the inhibition caused by PD98059 (Figure 6). We conclude that while part of the inhibitory effect of ERK2 inactivation on VSMC migration is to block PDGF-stimulated CamKII activation, an additional dominant CamKII-independent pathway of inhibition exists in ERK2-inhibited VSMCs.

Our previous studies established an important regulatory role for CamKII in PDGF-directed migration of VSMCs. Both PDGF-stimulated CamKII activity and PDGF-directed migration are impaired in differentiated VSMCs (Pauly *et al.*, 1995) and in VSMCs pretreated with a neutralizing antibody to

hesion of serum-starved VSMCs to 50 $\mu\text{g}/\text{ml}$ gelatin in the presence of 10 μM KN62 and/or 50 μM PD98059. In the first three bars, cells were pre-incubated with either KN62 or PD98059 and then transferred to the adhesion apparatus. Because pretreatment with PD98059 markedly inhibited adhesion to gelatin (bar 3), the effect of treating with PD98059 after cells had attached [indicated by 50p (preattached)] was tested (bars 4 and 5). Data are expressed as per cent adhesion relative to untreated controls. The data show that PD98059 did not inhibit cell attachment once the cells had attached in its absence.

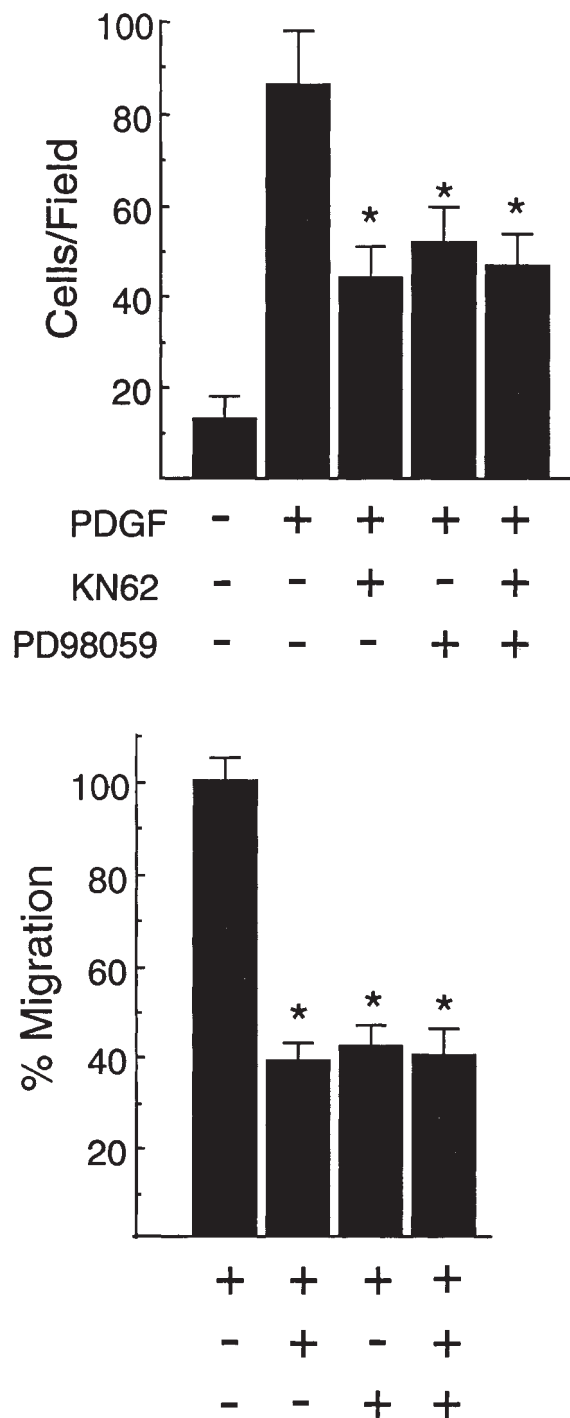


Figure 4 The combined effect of CamKII and ERK1/2 inhibition on PDGF-directed VSMC migration. The effect of the CamKII inhibitor, KN62 (10 μ M), and the MEK inhibitor, PD98059 (50 μ M), added alone or in combination on PDGF-directed migration of rat VSMCs on a fibronectin substrate. The upper panel shows the results expressed as absolute number of cells/high power field, while the lower graph expresses the data as per cent PDGF-mediated migration. *Significantly different from PDGF alone ($p > 0.001$).

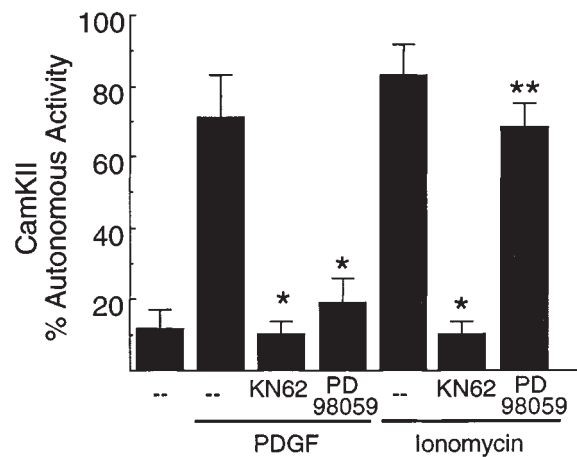


Figure 5 Effects of KN62 and PD98059 on CamKII activity. Serum-starved VSMCs were stimulated for 1 min with either 4 nM PDGF BB or 1 μ M ionomycin following pretreatment with either vehicle control (DMSO), 10 μ M KN62, or 50 μ M PD98059 and then assayed for CamKII activity as described in Methods. Activity is expressed as per cent autonomous activity. Both KN62 and PD98059 inhibit PDGF-stimulated CamKII activity, while only KN62 inhibits ionomycin-stimulated CamKII activity. *Significantly different from untreated stimulated control ($p > 0.001$).

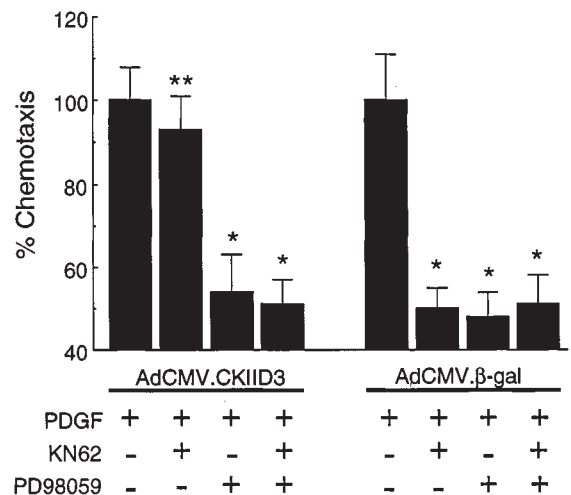


Figure 6 Forced expression of constitutively-activated CamKII rescues KN62-inhibited but not PD98059-inhibited VSMC migration toward PDGF. Effects of recombinant adenoviral infection of rat VSMCs with a virus expressing the cDNA for constitutively-activated CamKII (AdCMV.CKIID3) or *E. coli* β -galactosidase (AdCMV. β -gal). The effects of the inhibitors in the infected cells are expressed relative to PDGF-stimulated migration. *Significantly different from PDGF control ($p > 0.001$); **not significantly different from PDGF control ($p > 0.001$).

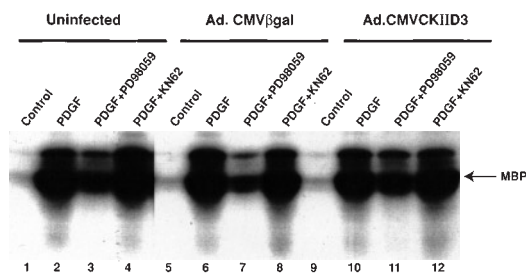


Figure 7 Effect of KN62 and PD98059 on MAPK activity in PDGF-stimulated VSMCs infected with adenoviruses AdCMV. β -gal and AdCMV.CKIID3. Phosphorylation of myelin basic protein (MBP) by the p42^{ERK2} immunocomplex from control (untreated), PDGF-stimulated, and PD98059 and KN62 treated serum-starved VSMCs.

bFGF (Bilato *et al.*, 1995) or blockers of $\alpha v\beta 3$ integrin–ligand interactions (Bilato *et al.*, 1997). Migration under all these conditions was fully restored through forced expression of constitutively activated CamKII. Growth state, bFGF and ligation of the $\alpha v\beta 3$ integrin complex are all factors that have been shown to influence VSMC migration *in vivo* (Lindner and Reidy, 1991; Lindner *et al.*, 1991; Choi *et al.*, 1994; Matumo *et al.*, 1994). CamKII, therefore, appears to be a focal point for integrating intracellular signaling events associated with migration of VSMCs in culture and *in vivo*. In contrast, we observed no effect on PDGF-stimulated ERK1/2 activation following blockade of the $\alpha v\beta 3$ integrin complex (Figure 8). Thus, unlike CamKII, ERK1/2 activation is apparently not an intracellular target for the $\alpha v\beta 3$ -mediated regulation of migration. This, however, does not discount the therapeutic potential for the treatment of vascular disorders centered around the inhibition of ERK1/2 activity, since inhibition has been shown to affect not only migration but proliferation of VSMCs as well (Nelson *et al.*, 1998).

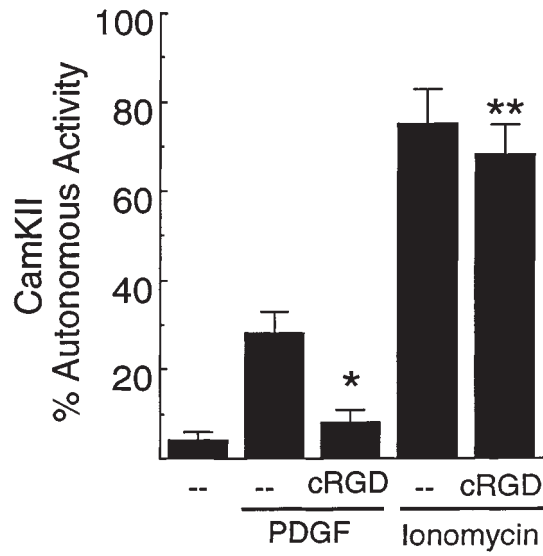
An unexpected outcome from these studies was the observation that ERK1/2 inhibition also inhibited PDGF-stimulated CamKII activity [Figure 5(B)]. Although PD98059 has been shown to be a very specific inhibitor of MEK (Alessi *et al.*, 1995), its direct effect on CamKII has not been reported. It seems unlikely, however, that the inhibition of PDGF-stimulated CamKII activity was due to a direct effect of PD98059 on CamKII, since ionomycin-induced CamKII activity was unaffected by PD98059. The mechanism by which PD98059 inhibits CamKII activation is unknown, although the differential effect of PDGF and ionomycin suggested that it may be related to intracellular calcium release. It has been reported, however, that PD98059 does not affect the peak levels of intracellular calcium release caused by PDGF (Graf

et al., 1997). The effect of PD98059 on CamKII activity may, therefore, be mediated by more subtle changes in intracellular calcium movement or possibly through the effects of phosphatases that function unopposed in ERK-inhibited VSMCs.

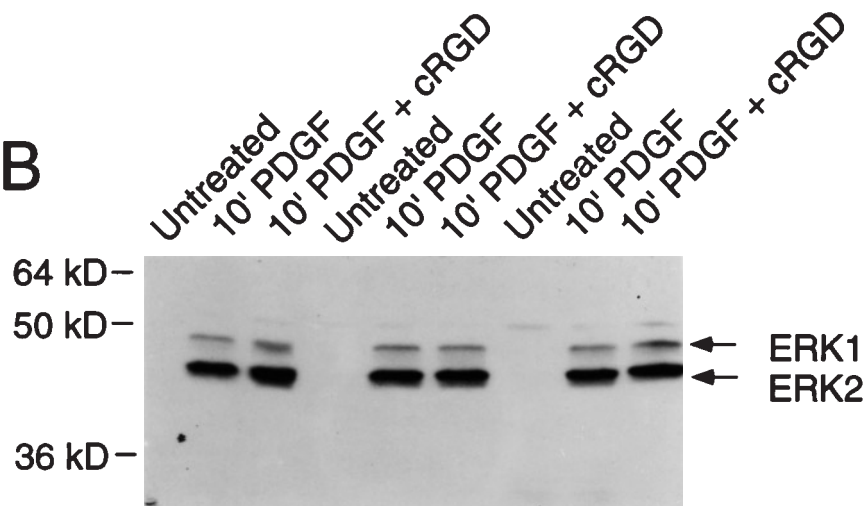
Although ERK1/2 activity has clearly been shown to be necessary for cell proliferation, their role in chemoattractant-directed migration has been controversial. Bornfeldt and colleagues (1994) reported that while both IGF and PDGF were effective chemoattractants for human VSMCs, IGF only weakly induced ERK activity in comparison to PDGF. Other studies demonstrated that while migration of cells expressing either dominant negative- or constitutively-activated RAS is suppressed, constitutively-activated RAF has no effect on migration, again suggesting that ERK activation (a downstream effect of RAF activation) was unimportant for migration (Kundra *et al.*, 1995). The availability of the MEK inhibitor, PD98059, has enabled a more direct evaluation of the role of ERK. Anand-Apte and colleagues (1997) reported that inhibition of ERK with PD98059 had no effect on PDGF-directed chemotaxis of Rat1 fibroblasts, although it did inhibit haptotaxis of these cells toward soluble fibronectin. Likewise, haptotaxis toward collagen type I was also shown to be inhibited by PD98059 (Klemke *et al.*, 1997). More recent studies using VSMCs have shown that PD98059 as well as antisense oligonucleotides against ERK1/2 effectively block PDGF-directed chemotaxis (Graf *et al.*, 1997; Nelson *et al.*, 1998), although we report here a partial reduction in chemotaxis rarely exceeding 70%. The reason for the difference between the experiments is not clear, but it should be noted that in both of the previous reports cited above, migrating VSMCs exposed to PDGF exhibited only a 3–4.5-fold increase over background levels. In our studies, the ratio of stimulated/background was at least 7–8-fold, almost double that of others.

How might inhibition of ERK affect migration? PDGF stimulation causes activated ERKs to translocate not only to the nucleus, but also to the plasma membrane and the cytoskeleton (Khalil and Morgan, 1993; Khalil *et al.*, 1995). It is not surprising then that likely targets of ERK activity would be proteins associated with specialized membrane structures, such as focal adhesion complexes, and the actin and microtubular cytoskeletal network. ERK1/2 associates with focal adhesion complexes after plating on various extracellular matrix substrates, consistent with a role in cell adhesion and migration (Miyamoto *et al.*, 1995). About half of all cellular MAPK activity generated by mitogenic stimulation also associates with microtubules

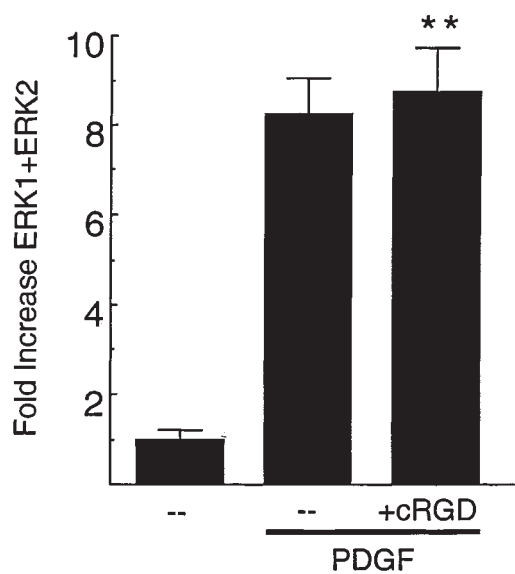
A



B



C



(Reszka *et al.*, 1995) and may be partially responsible for the observed correlations between MAPK activities and cytoskeletal alterations. Presumably, active MAPK travels to the microtubule and phosphorylates microtubule-associated proteins (MAP), such as tau, which is phosphorylated at 14 to 16 sites by MAPK (Drechsel *et al.*, 1992; Drews *et al.*, 1992). MAPK, a proline-directed kinase, may also catalyse caldesmon phosphorylation and this phosphorylation disinhibits the negative regulatory role on actin-activated myosin ATPase activity (Adam and Hathaway, 1993; Khalil *et al.*, 1995).

Myosin light chain kinase (MLCK) contains multiple MAPK consensus phosphorylation sites and ERK1 and ERK2 are capable of directly phosphorylating MLCK *in vitro*, resulting in increased enzymatic activity (Kumatsu and Hosoya, 1996; Klemke *et al.*, 1997). MLCK in non-muscle cells phosphorylates the regulatory myosin light chain (MLC) of myosin II leading to increased myofilament assembly and actin-activated ATPase activity (deLanerolle and Paul, 1991), events strongly implicated in directional cell movement. Klemke and colleagues (1997) have shown that elevated ERK activation through overexpression of constitutively activated MEK stimulates collagen-directed haptotaxis of COS7 cells along with increased phosphorylation of MLCK and MLCs. An inhibitor of MLCK, KT5962, not only blocks the MLC phosphorylation but also inhibits the increase in collagen-mediated haptotaxis resulting from MEK overexpression. It is interesting to note that CamKII phosphorylates MLCK at a site different from that phosphorylated by ERK1/2, having opposite effects on MLCK activity. Phosphorylation of MLCK by CamKII leads to inactivation of the enzyme, decreased MLC phosphorylation and disassembly of the smooth muscle myosin complex (Tansey *et al.*, 1992). In intact blood vessels, inactivation of MLCK by CamKII leads to vessel dilation. Whether a similar series of events occurs in VSMCs which have lost most of their differentiated phenotype and are capable of migrating is not known. If it did, it would

mean that both regulators (CamKII and ERK1/2) target the same effector molecule with opposite effects on its function. Given the importance that actomyosin interactions are likely to play in directed cell movements, the difference in actomyosin content at the advancing and trailing edges of a migrating cell may reflect differences either in regional localization or dominance of CamKII v ERK in the cell. Were the simultaneous activation and inhibition of MLCK required in different regions of a cell to produce directed movements, then the effects of inhibitors of these kinases regulating MLCK in opposite directions would be predicted to be non-additive, as reported here.

In summary, we have shown that inhibition of ERK activation following PDGF stimulation is as effective as CamKII inhibition in blocking PDGF-directed chemotaxis. Moreover, ERK inactivation affected chemotaxis through both CamKII-dependent and -independent pathways. Future studies are needed to focus on the exploitation of this signaling pathway in migrating VSMCs as a strategy for therapeutic intervention.

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Figure 8 Effects of the vitronectin receptor antagonist, cRGD, on PDGF-stimulated CamKII and ERK1/2 activation. (A) Serum-starved VSMCs were stimulated for 1 min with either 4 nM PDGF BB or 1 μ M ionomycin following pretreatment with either vehicle control (DPBS) or 30 μ g/ml GPenGRGDSPCA (cRGD) and then assayed for CamKII activity as described in Methods. Activity is expressed as per cent autonomous activity. cRGD inhibits PDGF- but not ionomycin-stimulated CamKII activity. *Significantly different from PDGF-stimulated control ($p > 0.001$). (B) Serum-starved VSMCs were stimulated for 10 min with 50 ng/ml PDGF BB following pretreatment with 30 μ g/ml GPenGRGDSPCA (cRGD). ERK activation was monitored with an antibody that recognizes dual threonine/tyrosine phosphorylation of ERK1/2 as described in Methods. Three different experiments are shown. (C) Quantitation of ERK phosphorylation. Results from densitometric analyses of three separate experiments. **Not significantly different from PDGF-stimulated sample.

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