1α,25-Dihydroxyvitamin D₃ Induces Vascular Smooth Muscle Cell Migration via Activation of Phosphatidylinositol 3-Kinase

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Abstract—The steroid hormone 1α,25-dihydroxyvitamin D₃ [1α,25-(OH)₂D₃] promotes vascular smooth muscle cell (VSMC) growth and calcification, but the precise mechanism by which 1α,25-(OH)₂D₃ regulates VSMC migration is unknown. In rat aortic SMCs, we found that 1α,25-(OH)₂D₃ (0.1 to 100 nmol/L) induced a dose-dependent increase in VSMC migration. This response required the activation of phosphatidylinositol 3-kinase (PI3 kinase) because 1α,25-(OH)₂D₃–induced migration was completely abolished by the PI3 kinase inhibitors, LY294002 (10 μmol/L) or wortmannin (30 nmol/L). Furthermore, the RNA polymerase inhibitor, 5,6-dichlorobenzimidazole riboside (50 μmol/L), did not affect 1α,25-(OH)₂D₃–induced VSMC migration, suggesting that gene transcription is not involved in this rapid response. Using analogs of 1α,25-(OH)₂D₃, which have been characterized for their abilities to induce either transcriptional or nontranscriptional responses of 1α,25-(OH)₂D₃, we found that 1α,25-dihydroxylumisterol, which is a potent agonist of the rapid, nongenomic responses, was equipotent with 1α,25-(OH)₂D₃ in inducing PI3 kinase activity and VSMC migration. Moreover, 1β,25-(OH)₂D₃, which specifically antagonizes the nongenomic actions of 1α,25-(OH)₂D₃, abolished 1α,25-(OH)₂D₃–induced PI3 kinase activity and VSMC migration, whereas the inhibitor of the genomic actions of vitamin D, (23S)-25-dehydro-1α-OH-D₃-26,23-lactone, did not affect these responses. These results indicate that 1α,25-(OH)₂D₃ induces VSMC migration independent of gene transcription via PI3 kinase pathway, and suggest a possible mechanism by which 1α,25-(OH)₂D₃ may contribute to neointima formation in atherosclerosis and vascular remodeling. (Circ Res. 2002;91:17-24.)

Key Words: steroid hormones • smooth muscle • migration • phosphatidylinositol 3-kinase
Recent studies suggest that 1α,25-(OH)2D3 plays an important role in the cardiovascular system through its receptors in the heart and in vascular smooth muscle cells (VSMCs). In particular, 1α,25-(OH)2D3 has been shown to regulate calcium homeostasis, modulate growth, and increase calcification in smooth muscle cells. Indeed, the mitogenic role of 1α,25-(OH)2D3 in VSMCs has been previously reported, although its effect on the migration has not been investigated. Therefore, the purpose of this study was to determine whether 1α,25-(OH)2D3 can promote SMC migration, and if so, to determine whether the mechanism is mediated by the genomic or nongenomic effects of VDR.

Materials and Methods

Materials

1α,25-(OH)2D3 was purchased from Biomol Research Laboratories Inc. 1α,25-(OH)2D3–lumisterol (JN), 1β,25-(OH)2D3 (HL), and (23S)-25-dehydro-1α-OH-Dβ26,23-lactone (MK) were generously provided by A.W. Norman and W.H. Okamura (University of California, Riverside, Calif).

Cell Culture

Rat aortic VSMCs were harvested from Sprague-Dawley rats (Taconic Farms, Germantown, NY) by enzymatic dissociation according to the method of Gunther et al. Cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc) supplemented with 10% fetal calf serum (Hyclone), penicillin (100 U/mL), streptomycin (100 U/mL), and fungizone (1.25 μg/mL). Cellular viability under the experimental conditions was determined by cell number, cellular morphology, and trypan blue exclusion. The guidelines on handling and care of animals were approved by our institutional Animal Care Committee.

Migration Assay

VSMC migration was examined in modified Boyden transwell cell culture chambers using a gelatin-treated polycarbonate membrane with 8-μm pores in 24-well plates (Costar Inc). Cells were grown to 80% confluence and starved for 48 hours in DMEM containing 0.4% charcoal-stripped FCS. After starvation cells were trypsinized, counted, and resuspended in serum-free DMEM to a concentration of 2×10⁶ cells/mL. Cells (100 μL) were then added to the upper chamber, while the lower chamber was filled with 600 μL of DMEM containing the appropriate concentration of agonist or vehicle. After 4 hours incubation at 37°C, cells were removed from the upper side of the membranes with cotton swabs. The membranes were then fixed with methanol (10 minutes at 4°C) and stained with hematoxylin. The number of migrated cells was counted under microscope (magnification, 400×). Three randomly chosen fields were counted per membrane, and each experiment was performed in triplicate.

Adhesion Assay

VSMC adhesion was assessed in transwell cell culture chambers after a procedure similar to that described above for the migration assay. Briefly, cells were resuspended to a concentration of 2×10⁴ cells/mL, and 2×10⁴ cells were added to the upper chamber. DMEM containing the appropriate concentration of agonist or vehicle was added to the lower chamber. After 2 hours incubation at 37°C in a 5% CO₂ incubator, the media containing nonadherent cells was removed, and cells fixed on the upper surface of the membranes were counted. Experiments were performed in triplicate.

Phosphatidylinositol 3-Kinase Assay

Phosphatidylinositol 3-kinase (PI3 kinase) activity in VSMC lysates was assayed using the borate thin layer chromatography method as described. After the indicated treatment conditions in DMEM containing 0.4% charcoal-stripped FCS, cells were harvested in ice-cold lysis buffer (in mmol/L: 137 NaCl, 20 Tris-HCl [pH 7.4], 1 CaCl₂, 1 MgCl₂, 0.1 Na,VO₃, 1% NP-40). After pelleting the cell debris, the supernatant was incubated for 1 hour at 4°C with 5 μL of a VDR antibody (recognizes C-terminus, ligand-binding domain, 1 μg; Santa Cruz Biotechnology) and immunoprecipitated with the addition of 50 μL of a 1:1 slurry of protein A-agarose for 1 hour at 4°C. After centrifugation, the immunoprecipitates were washed 3 times with lysis buffer, 3 times with 0.1 mol/L Tris-HCl, pH 7.4, 5 mmol/L LiCl, and 0.1 mmol/L Na,VO₃, followed by 2 washes with 80% confluence and starved for 48 hours in DMEM containing 0.4% charcoal-stripped FCS. After starvation cells were trypsinized, counted, and resuspended in serum-free DMEM to a concentration of 2×10⁶ cells/mL. Cells (100 μL) were then added to the upper chamber, while the lower chamber was filled with 600 μL of DMEM containing the appropriate concentration of agonist or vehicle. After 4 hours incubation at 37°C, cells were removed from the upper side of the membranes with cotton swabs. The membranes were then fixed with methanol (10 minutes at 4°C) and stained with hematoxylin. The number of migrated cells was counted under microscope (magnification, 400×). Three randomly chosen fields were counted per membrane, and each experiment was performed in triplicate.

Figure 1. Structure of the steroid hormone 1α,25-(OH)2D₃ and related analogs. 1α,25-(OH)2D₃ has 360° rotational flexibility around the 6,7 single carbon bond; the two extreme conformers, the steroid-like 6-s-cis and the extended 6-s-trans are shown. This 360° rotation around the 6,7 carbon bond, which is a reflection of the chemical properties of the single carbon bond, occurs millions of times per second and generates a continuum of shapes that are available for ligand binding to any vitamin D–related receptor(s) that may be present. Thus, the conformationally flexible 1α,25-(OH)2D₃ is able to generate shapes that satisfy the receptor requirements both for genomic responses (VDR) as well as rapid nongenomic responses. In contrast, the analog 1α,25-(OH)2D₃–lumisterol (JN) has a 9,10 carbon bond that locks it exclusively in the 6-s-cis shape with the consequence that it is a full agonist only for rapid nongenomic responses, but is not an agonist for genomic responses. The analog 1β,25-(OH)2D₃ (HL) is an antagonist for only rapid nongenomic responses. The compound (23S)-25-dehydro-1α-OH-Dβ26,23-lactone (MK) is an antagonist of only genomic responses.
(in mmol/L) 10 Tris-HCl (pH 7.40), 150 NaCl, 5 mmol/L EDTA, and 0.1 NaVO₃.

The immunoprecipitates were then mixed with 50 μL Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 20 μg of phosphatidylinositol-3,4-bisphosphate (PI₃, Sigma), 10 μL 100 mmol/L MgCl₂, and 5 μL of a 0.88 mmol/L ATP, 20 mmol/L MgCl₂ solution, containing 30 μCi of [γ-³²P]ATP (3000 Ci/mmol; NEN Life Science Products). The reaction was incubated at 37°C for 10 minutes, and subsequently blocked by the addition of 20 μL of 6N HCl. The phospholipids were extracted with 160 μL of chloroform/methanol (1:1, v/v). The organic phase (50 μL), containing the labeled PI3 kinase products, was separated by borate thin layer chromatography on glass-backed Silica Gel 60 plates (EM Separations) pretreated with a solution containing 25 mmol/L trans-1,2-diaminocyclohexane-N,N,N,N’-tetraacetic acid (CDTA, Sigma), 66% (v/v) ethanol, and 0.06 N NaOH, dried for 1 hour and then baked at 100°C for 15 minutes. The chromatography was developed with a solution containing 37.5% (v/v) methanol, 30% (v/v) chloroform, 22.5% (v/v) pyridine (Sigma), 1.33% (v/v) formic acid, 1 mol/L boric acid and 8.5 mol/L butylated hydroxytoluene (Sigma), briefly dried, and exposed to autoradiography.

**Western Blotting**

After 24 hours stimulation in DMEM containing 0.4% charcoal-stripped FCS, VSMCs were washed with ice-cold PBS and lysed with 500 μL of the following buffer: Tris-HCl (20 mmol/L, pH 7.4), EDTA (10 mmol/L), NaCl (100 mmol/L), IGEPAL (1%), Na₂VO₃ (1 mmol/L), NaF (50 mmol/L) containing a cocktail of protease inhibitors (Roche). Proteins (20 μg) were separated by SDS-PAGE on a 10% acrylamide gel and blotted onto nitrocellulose membrane (Osmonics). Afterward, membranes were incubated for 2 hours at room temperature with antibodies raised against osteopontin (Santa Cruz Biotech.) or α-tubulin (DM1A, Sigma). Then, the blots were washed and incubated for 1 hour with horseradish peroxidase-labeled anti-goat (Santa Cruz Biotech.) or anti-mouse (Amersham Pharmacia) antibodies, respectively. Immunoreactive bands were visualized with a chemiluminescence kit (PerkinElmer Life Science) and quantified by densitometry.

**Statistical Analysis**

All values are expressed as mean±SEM. Differences between groups were determined by the unpaired, 2-tailed Student’s t test. Values of P<0.05 were accepted as statistically significant.

**Results**

**Cell Culture**

Relatively pure (>95%) rat aortic SMCs were confirmed by their morphological features using phase-contrast microscopy and immunofluorescent staining with a smooth muscle–specific MHC antibody (data not shown). There were no observable adverse effects of wortmannin, LY294002, or 1,25-dihydroxyvitamin D₃ on cellular viability for all experimental conditions, except when given for >12 hours (ie, trypan blue exclusion is 85% to 90% versus >95% for <12 hours).

**PI3 Kinase Mediates 1α,25-Dihydroxyvitamin D₃–Induced VSMC Migration**

The effect of 1α,25-dihydroxyvitamin D₃ on VSMC migration was assessed using a modified Boyden transwell cell culture chamber. The addition of 1α,25-(OH)₂D₃ in the lower compartment of the transwells induced a concentration-dependent increase of VSMC migration. Indeed, physiological 1α,25-(OH)₂D₃ concentrations ranging from 0.1 to 100 nmol/L increased VSMC migration by 19% to 42% over control (P<0.05, n=3; Figure 2A). Because migration is, in part, dependent on the adhesion of the VSMCs to the transwell membrane, we tested whether 1α,25-(OH)₂D₃ had any effect on VSMC adhesion. As shown in Figure 2B, the number of cells attached to the upper side of the transwell membrane was not affected by 1α,25-(OH)₂D₃.

A role for PI3 kinase in VSMC migration has been previously reported. Therefore, we investigated whether 1α,25-(OH)₂D₃–induced VSMC migration was dependent on PI3 kinase activity. Studies were performed using two selective inhibitors of PI3 kinase: wortmannin and LY294002. Preincubation of VSMCs for 30 minutes with wortmannin (30 mmol/L) or LY294002 (10 μmol/L), which selectively inhibits PI3 kinase, abolished the VSMC migration induced by 10 nmol/L of 1α,25-(OH)₂D₃ (P<0.05, n=3) (Figure 3). These results suggest that 1α,25-(OH)₂D₃ increases the migration of VSMCs and that this response requires the activation of PI3 kinase.

**Activation of PI3 Kinase by 1α,25-(OH)₂D₃ in VSMCs**

To further support the hypothesis that PI3 kinase is involved in 1α,25-(OH)₂D₃–induced VSMC migration, we tested whether 1α,25-(OH)₂D₃ can induce PI3 kinase activity. As shown in Figure 4A, stimulation of VSMCs with 1α,25-
(OH)\(_2\)D\(_3\) (10 nmol/L) induced a rapid time-dependent increase in VDR-associated PI3 kinase activity. Indeed, after 10 and 30 minutes, 1\(\alpha\),25-(OH)\(_2\)D\(_3\) (1 nmol/L) increased PI3 kinase activity by 5- and 10-fold, respectively. In agreement with the results obtained in our migration assays, the dose-response experiments revealed an increase in PI3 kinase activity using 1\(\alpha\),25-(OH)\(_2\)D\(_3\) concentrations as low as 0.1 nmol/L (P<0.05, n=3; Figure 4B). The 1\(\alpha\),25-(OH)\(_2\)-induced PI3 kinase activity was abolished in cells pretreated with unstimulated or control cells (C). B, Effect of increasing concentrations of 1\(\alpha\),25-(OH)\(_2\)D\(_3\) on PI3 kinase activity. Cells were stimulated for 10 minutes with 1\(\alpha\),25-(OH)\(_2\)D\(_3\) (0.01 to 10 nmol/L) or left untreated (C). Effect of wortmannin (Wort, 30 nmol/L), LY294002 (LY, 10 \(\mu\)mol/L) or DRB (50 \(\mu\)mol/L) on PI3 kinase activity induced by 1\(\alpha\),25-(OH)\(_2\)D\(_3\) (10 nmol/L). All experiments were performed 3 times with comparable results.

**Nontranscriptional Effect of 1\(\alpha\),25-(OH)\(_2\)D\(_3\) on VSMC Migration**

1\(\alpha\),25-(OH)\(_2\)D\(_3\) has been shown to generate biological responses via both genomic and rapid nongenomic pathways. To determine whether 1\(\alpha\),25-(OH)\(_2\)D\(_3\)-induced migration involves gene transcription, VSMCs were treated for 30 minutes with the RNA polymerase inhibitor, 5,6-dichlorobenzimidazole riboside (DRB, 50 \(\mu\)mol/L) before 1\(\alpha\),25-(OH)\(_2\)D\(_3\) stimulation. DRB did not affect 1\(\alpha\),25-(OH)\(_2\)D\(_3\)-induced VSMC migration or PI3 kinase activity, suggesting that gene transcription is not involved in these responses (Figures 3 and 4C). Indeed, treatment of VSMCs with DRB inhibited TNF-\(\alpha\)-induced gene transcription by >95% using nuclear run-on analyses (data not shown).

**VSMC Migration Mediated by Stereospecific Activation of VDR**

The 6-s-cis–locked, 1\(\alpha\),25-dihydroxyvitamin D\(_3\) analog, 1\(\alpha\),25-(OH)\(_2\)-lumisterol (JN), has been shown to be the most effective agonist of the rapid nongenomic responses characteristic of 1\(\alpha\),25-dihydroxyvitamin D\(_3\). Therefore, we used this analog to further study the hypothesis that the migration of VSMCs induced by 1\(\alpha\),25-dihydroxyvitamin D\(_3\) is a nongenomic response. Similarly to 1\(\alpha\),25-(OH)\(_2\)D\(_3\), 1 and 10 nmol/L of JN significantly increased VSMC migration by 28% and 41%, respectively (P<0.05, n=3; Figure 5A). Moreover, the same analog induced a rapid, dose-dependent increase in PI3 kinase activity (Figures 5B and 5C). Indeed, after 10 minutes stimulation with 1 and 10 nmol/L of JN, PI3 kinase activity was increased approximately by 4.5- and 11-fold, respectively. To verify that JN does not induce VDRE-dependent responses in VSMCs, we measured the expression of osteopontin, a protein induced by 1\(\alpha\),25-(OH)\(_2\)D\(_3\) in SMCs. Stimulation with 1\(\alpha\),25-(OH)\(_2\)D\(_3\) increased the amount of osteopontin protein in VSMCs by approximately 2-fold (P<0.05, n=3), which was completely blocked by cotreatment with the transcriptional inhibitor, DRB (50 \(\mu\)mol/L; Figure 6). Consistent with its established nongenomic action, JN did not affect osteopontin expression in VSMCs. Our results indicate that the JN analog is equipotent with 1\(\alpha\),25-(OH)\(_2\)D\(_3\) in inducing PI3 kinase activation and migration in VSMCs and support the hypothesis that gene transcription is not involved in these responses.

We next tested two specific antagonists of the VDR. 1\(\beta\),25-(OH)\(_2\)D\(_3\) (HL) has been shown to be a potent antago-
increased VSMC migration with significant responses observed above 0.1 nmol/L. This concentration is comparable with the physiological level of total circulating 1α,25-(OH)2D3.30 Furthermore, much higher local concentrations of 1α,25-(OH)2D3 could be reached under pathophysiological conditions. Indeed, monocytes and macrophages have been shown to produce 1α,25-(OH)2D3 in a paracrine manner after stimulation with inflammatory stimuli.31−33 Our observation that 1α,25-(OH)2D3-induced VSMC migration requires PI3 kinase activation is in agreement with the role of PI3 kinase in cell migration processes regulated by cytokines and growth factors in diverse cell types including VSMCs.34−36 We found an induction of VDR-associated PI3 kinase activity by 1α,25-(OH)2D3 in VSMCs. This observation is consistent with that reported by Hmama et al37 in myeloid cells.

Our findings further indicated that the VSMC migration induced by 1α,25-(OH)2D3 was a rapid response that is independent of VDRE-regulated gene transcription. For instance, the RNA polymerase inhibitor DRB did not affect 1α,25-(OH)2D3-induced VSMC migration and 1α,25-(OH)2D3 rapidly induced (ie, within 10 minutes) PI3 kinase activity that correlated with VSMC migration. Furthermore, we found that the 1α,25-(OH)2D3 6-s-cis−locked analog JN, which has been reported to specifically induce the non-genomic responses characteristic of 1α,25-(OH)2D3 in several cell types,5,38 mimicked both 1α,25-(OH)2D3−induced PI3 kinase activation and VSMC migration. Finally, the 1α,25-(OH)2D3 antagonist HL, which specifically blocks the non-genomic responses of 1α,25-(OH)2D3,8,13,38 inhibited both 1α,25-(OH)2D3−induced PI3 kinase activation and VSMC migration

**Discussion**

In the present study, we showed that 1α,25-(OH)2D3 induced a dose-dependent increase in VSMC migration with significant responses observed above 0.1 nmol/L. This concentration is comparable with the physiological level of total circulating 1α,25-(OH)2D3.30 Furthermore, much higher local concentrations of 1α,25-(OH)2D3 could be reached under pathophysiological conditions. Indeed, monocytes and macrophages have been shown to produce 1α,25-(OH)2D3 in a paracrine manner after stimulation with inflammatory stimuli.31−33 Our observation that 1α,25-(OH)2D3−induced VSMC migration requires PI3 kinase activation is in agreement with the role of PI3 kinase in cell migration processes regulated by cytokines and growth factors in diverse cell types including VSMCs.34−36 We found an induction of VDR-associated PI3 kinase activity by 1α,25-(OH)2D3 in VSMCs. This observation is consistent with that reported by Hmama et al37 in myeloid cells.

Our findings further indicated that the VSMC migration induced by 1α,25-(OH)2D3 was a rapid response that is independent of VDRE-regulated gene transcription. For instance, the RNA polymerase inhibitor DRB did not affect 1α,25-(OH)2D3−induced VSMC migration and 1α,25-(OH)2D3 rapidly induced (ie, within 10 minutes) PI3 kinase activity that correlated with VSMC migration. Furthermore, we found that the 1α,25-(OH)2D3 6-s-cis−locked analog JN, which has been reported to specifically induce the non-genomic responses characteristic of 1α,25-(OH)2D3 in several cell types,5,38 mimicked both 1α,25-(OH)2D3−induced PI3 kinase activation and VSMC migration. Finally, the 1α,25-(OH)2D3 antagonist HL, which specifically blocks the non-genomic responses of 1α,25-(OH)2D3,8,13,38 inhibited both 1α,25-(OH)2D3−induced PI3 kinase activation and VSMC migration.

**Figure 5.** 1α,25-(OH)2-lumisterol (JN) induces VSMC migration and PI3 kinase activity. A, Effect of JN (1 and 10 nmol/L) on VSMC migration. Experiments were performed 3 times in triplicates. *P<0.05 compared with unstimulated cells. B, Time-dependent (10 to 30 minutes) increase in PI3 kinase activity induced by JN (1 nmol/L) compared with unstimulated cells (C). C, Effect of increasing concentrations of JN on PI3 kinases activity. Cells were stimulated for 10 minutes with JN (1 to 100 nmol/L) or left untreated (C). Experiments were performed 3 times with comparable results.

**Figure 6.** Effect of 1α,25-dihydroxyvitamin D3 and JN on osteopontin expression in VSMCs. A, Cells were stimulated for 24 hours with 1α,25-(OH)2D3 (1 nmol/L), JN (1 nmol/L), or left untreated (Control) in the presence or absence of DRB (50 μmol/L). Osteopontin (OPN) protein level was detected by Western blotting and equivalent loading was standardized to that of α-tubulin. B, Immunoreactive bands corresponding to OPN were quantitated by densitometric analysis. Blots are representative of results obtained in three separate experiments. *P<0.05 compared with unstimulated cells (Control).
migration. These findings suggest that there may exist a rapid-response membrane receptor that mediates the non-
genomic actions of 1α,25-(OH)2D3. However, because the cis to trans configuration of 1α,25-(OH)2D3 occurs fairly rapidly and has not been shown to be affected under pathophysiologi-
ical conditions, it is unlikely that changes in the ratio of cis to trans isoform contribute to the diversity of SMC response to 1α,25-(OH)2D3. Rather it is probably due to the “dual” ligand nature of 1α,25-(OH)2D3 and the phenotypic state of SMC, with the cis isoform eliciting SMC migration via the PI3 kinase pathway.

The mechanism underlying this nongenomic response is not completely understood. Our PI3 kinase assays indicate that a signaling complex containing both the VDR and PI3 kinase is formed after 1α,25-(OH)2D3 stimulation. The identity and characterization of the VDR involved in this nongenomic response remains to be determined. However, the antibody used in our assay recognizes the C-terminal region of the nuclear VDR, suggesting that the classical nuclear VDR mediates PI3 kinase activation rather than a novel membrane receptor. A similar observation was reported concerning the nongenomic effects of estrogen in endothelial cells.39 Indeed, the estrogen receptor was shown to induce PI3 kinase activation by binding to the PI3 kinase regulatory subunit p85α. Also Kousteni et al40 have described nontradi-
tional results for both the estrogen and androgen nuclear receptors in osteoblasts, where they are shown to be involved in activation of a Src/Shc/ERK signaling pathway and rapid attenuation of apoptosis. These actions were mediated by nuclear receptors, under circumstances that eliminated nuclear targeting of the receptor proteins. Further, they demon-
strated that the antiapoptotic rapid action could be dissociated from the transcriptional activity of the receptors by use of synthetic ligands that are specific for either genomic or rapid actions. This is analogous to our use of 6-s-cis–locked analogs of 1α,25-(OH)2D3.

The present results also demonstrate that the 6-s-cis–
locked analog JN is a potent agonist of the signaling nuclear VDR/PI3 kinase complex and implies that the nuclear VDR can productively interact with this ligand. However, this conclusion is at variance with the weak affinity of JN for the nuclear VDR in a simple in vitro binding study (<0.5%)5 or in settings where the nuclear VDR (occupied by JN) is interacting with VDRE to initiate classic genomic responses (<1.5%),5 both in comparison to 1α,25-(OH)2D3. Thus, the present PI3 kinase system affords an unusual opportunity to decipher and understand the how the nuclear VDR in the presence of 6-s-cis–shaped analogs mediates productive bio-
logical responses. Interestingly, Baran et al have recently shown that annexin II can bind 1α,25-(OH)2D3 as a mem-
brane receptor and mediate the rapid responses of 1α,25-(OH)2D3 on calcium influx.41,42 Although we have shown that VDR can associate with PI3 kinase in a ligand-dependent manner, similar findings have not been reported for annexin II. Thus, it is possible that VDR and annexin can both function as membrane receptors, which mediate distinct, nongenomic actions of 1α,25-(OH)2D3.

A possible downstream target of PI3 kinase in 1α,25-
(OH)2D3–induced VSMC migration is the focal adhesion kinase (FAK), which is a cytoplasmic tyrosine kinase that plays a crucial role in the migration process.43 Because PI3 kinase activity has been shown to be required for PDGF-
induced FAK phosphorylation,44 it is possible that a similar pathway is activated by 1α,25-(OH)2D3. On the other hand, Src kinases have also been shown to induce FAK phosphory-
ation and, therefore, play a role in cell migration.45 In this 
context, the nuclear VDR has also been shown to activate tyrosine phosphorylation pathways.46,47 In particular, 1α,25-(OH)2D3 has been reported to induce Src activation and association to tyrosine phosphorylated VDR.48 As it has recently been shown for the estrogen receptor (ER),49 it is possible that a complex consisting of VDR, Src, and PI3 kinase may form after 1α,25-(OH)2D3 stimulation, mediate the subsequent phosphorylation of FAK, and increase cell migration. However, the subcellular distribution of ER differs from VDR, and therefore, the mechanism by which VDR activates PI3 kinase may be very different from that of ER. Future studies aimed to elucidate the potential role of Src and FAK in 1α,25-(OH)2D3–induced VSMC migration will help identify the possible interaction between these proteins and the VDR response in the vascular wall.
Acknowledgments

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