Report

Adhesion-dependent activation of CaMKII and regulation of ERK activation in vascular smooth muscle

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Lu, Katherine Kun, Shayn E. Armstrong, Roman Ginnan, and Harold A. Singer. Adhesion-dependent activation of CaMKII and regulation of ERK activation in vascular smooth muscle. *Am J Physiol Cell Physiol* 289: C1343–C1350, 2005. First published June 15, 2005; doi:10.1152/ajpcell.00064.2005.—Cell adhesion-dependent activation of ERK1/2 has been linked functionally to focal adhesion dynamics. We previously reported that in adherent vascular smooth muscle (VSM) cells, CaMKII mediates ERK1/2 activation in response to Ca^{2+}-mobilizing stimuli. In the present study, we tested whether CaMKII regulates ERK1/2 signaling in response to VSM cell adhesion. Using an antibody that specifically recognizes CaMKII autophosphorylated on Thr^{287}, we determined that CaMKII is rapidly activated (within 1 min) after the adherence of cells on multiple ECM substrates. Activation of CaMKII on fibronectin was unaffected in cells overexpressing focal adhesion kinase (FAK)-related nonkinase (FRNK), an endogenous inhibitor of FAK. Furthermore, CaMKII was rapidly and robustly activated in VSM cells plated on poly-L-lysine. These results suggest that adhesion-dependent CaMKII activation is integrin independent. Adhesion-dependent FAK activation on fibronectin was not affected in cells treated with the selective CaMKII inhibitor KN-93 (30 μM) or in cells in which the expression of CaMKII with small interfering RNA (siRNA) was suppressed, although tyrosine phosphorylation of paxillin was inhibited in CaMKII-δ_2-suppressed cells. Sustained ERK1/2 activation that was dependent on FAK activation (inhibited by FRNK) was also attenuated by CaMKII inhibition or siRNA-mediated gene silencing. Rapid ERK1/2 activation that preceded FAK and paxillin activation was detected upon VSM cell adhesion to poly-L-lysine, and this response was inhibited by CaMKII gene silencing. These results indicate that integrin-independent CaMKII activation is an early signal during VSM cell adhesion that positively modulates ERK1/2 signaling through FAK-dependent and FAK-independent mechanisms.

calcium/calmodulin-dependent protein kinase II; cell adhesion; extracellular signal-regulated protein kinases; paxillin; focal adhesion kinase

ACTIVE ERK1/2 is involved in the regulation of several basic cellular functions such as cell proliferation, migration, and gene expression (15, 35, 40). Activation of these functions in vascular smooth muscle (VSM) in response to injury or disease contributes significantly to vascular pathology, resulting in restenosis and atherosclerotic lesion formation (19). Diverse stimuli are capable of activating ERK1/2, including receptor tyrosine kinase-coupled growth factors, G protein-coupled receptor (GPCR) ligands, and integrin engagement (1, 5, 7, 35). ERK1/2 activation is generally reported to require the sequential activation of Ras, Raf, and MEK, but a number of signaling pathways may be used proximal to Ras activation in a stimulus- and cell-specific manner (11, 35). Substantial evidence has been produced indicating spatial organization of signaling intermediates in the ERK activation cascade facilitated by scaffolding proteins and/or scaffolding domains in receptor tyrosine kinases (4, 35). In cultured VSM cells, we have documented PKC δ-isozyme and CaMKII pathways that mediate GPCR ligand-dependent activation of ERK1/2 (1, 10, 11). PKC-δ and CaMKII-dependent ERK activation in VSM requires activation of nonreceptor tyrosine kinases (Src family kinases and Pyk2) and transactivation of epidermal growth factor receptors (EGFRs) (10, 11).

Cell adhesion to extracellular matrix proteins such as fibronectin and collagen is mediated by the binding of integrin receptors to ECM ligands (13). Integrin engagement leads to a number of intracellular signaling events, including the activation of Src family kinases (SFKs) and ERK1/2, responses that are dependent on the tyrosine phosphorylation (Tyr^{397}) and activation of focal adhesion kinase (FAK) (20). A potential mechanism for integrin-dependent activation of ERK1/2 involves the scaffolding protein paxillin, which is activated by binding to FAK and Src-dependent tyrosine phosphorylation upon integrin clustering (4, 12). Activated paxillin binds Raf, MEK, and ERK, thereby facilitating ERK activation and localization in focal adhesions (4, 37). The importance of paxillin in promoting adhesion-dependent ERK1/2 activation has been indicated in studies documenting diminished adhesion-dependent ERK activation in paxillin-knockout cells (12). FAK, SFKs, paxillin, and ERK1/2 are key regulators of focal adhesion dynamics and therefore are important in regulating cell adhesion and migration (22, 37).

Intracellular Ca^{2+} signals can also initiate events that are important in regulating VSM cell migration (28, 33). One possible mechanism is disassembly of focal adhesions at the trailing edge of migrating cells in response to localized transient increases in free intracellular Ca^{2+} (9). A combination of cell adhesion and serum or growth factor stimulation has reported to increase the frequency of cultured VSM cell Ca^{2+} transients as well as the rate of migration (28). Integrin signaling may play a role in regulating intracellular Ca^{2+} (31, 38). It has been reported that ligand binding of integrin receptors triggered Ca^{2+} influx and inositol 1,4,5-trisphosphate (IP_3)-dependent intracellular Ca^{2+} release in Madin-Darby canine kidney epithelial cells (31). In differentiated contractile VSM cells, integrin $\alpha_v\beta_3$ and $\alpha_5\beta_1$ differentially regulate L-type Ca^{2+} channels, which is related to Ca^{2+} influx leading to muscle contraction (38). Conversely, CaM-dependent activation of calcineurin and CaMKII has been reported to regulate integrin binding affinity (3). Several studies have implicated

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CaMKII as a mediator of Ca^{2+}-dependent regulation of VSM cell migration (2, 18, 23, 24), although the targets and mechanisms of regulation are not yet clear. In a recent study in which the researchers used an immortalized thyroid cell line (TAD-2) and Hep3B cells, adhesion to fibronectin was found to activate ERK1/2 by a CaMKII- and Raf-dependent mechanism (14).

In the present study, we first tested the hypothesis that CaMKII is activated upon VSM cell adhesion and determined the role of integrin-dependent signaling pathways involving FAK activation in that process. The studies indicated that VSM cell adhesion results in rapid activation of CaMKII by a mechanism that does not require integrin signaling or FAK activation. Second, using both pharmacological and molecular approaches, we tested the function of CaMKII in modulating adhesion-dependent signaling events that result in ERK1/2 activation. Adhesion-dependent activation of CaMKII does not affect FAK activation but does facilitate FAK-dependent tyrosine phosphorylation of paxillin and ERK1/2 activation. Overall, the results suggest that adhesion-dependent activation of CaMKII and FAK are independent events and that CaMKII facilitates ERK1/2 activation through both integrin-dependent and integrin-independent mechanisms. CaMKII-dependent regulation of paxillin and ERK1/2 activation is a potential mechanism affecting focal adhesion dynamics.

METHODS

Cell culture. VSM cells were obtained from the medial layer of the thoracic aortas of 200- to 300-g Sprague-Dawley rats as described previously (8). After removal of the adventitial and endothelial layers, the medial VSM cells were enzymatically dispersed and cultured in DMEM-Ham's F-12 medium with 10% fetal bovine serum (FBS). The VSM cells were maintained at 37°C in a 5% CO_2 atmosphere and split twice weekly. The cells from *passages 3–10* were used for experiments. The use of experimental animals for these procedures was reviewed and approved by the Albany Medical College Institutional Care and Use Committee.

SiRNA targeting CaMKII δ-isoforms and adenovirus construction. The siRNA Target Finder and Design Tool (Ambion, Austin, TX) was used to select specific sequences targeting the rat CaMKII δ-isoforms (GenBank accession no. NM_12519). Potential small interfering RNA (siRNA) targets were subjected to BLAST searches against expressed sequence tag libraries to confirm specificity and multispecies identity. Target sequences were used to generate adenoviruses expressing short hairpin RNA (shRNA) targeting the CaMKII δ-subunit, the principal isoform expressed in cultured VSM cells (29). The sequence targeting all CaMKII δ-isoforms used in the present study was 5′-AAGACAT-AGTGGCAAGAGAAT-3′, nt 521–541, and is referred to as siDELTA.

To obtain high efficiency of silencing in these cells, adenoviruses expressing shRNA were produced using the pSuppressorAdeno system (IMG-1200; Imgenex, San Diego, CA). Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The sense strand for siDELTA was 5′-TCGAGACATAGTGGCAA-GAGAATAT<u>ATCGAT</u>ATATTCTCTTGCCACTATGTC-3, and the antisense strand was 5′-CTAGGACATAGTGGCAAGAGAATAT-<u>ATCGAT</u>ATATTCTCTTGCCACTATGTC-3′. The underlined nucleotides represent the loop sequence. Annealed DNA was ligated into the linearized pSuppressorAdeno "shuttle" vector and then transformed into competent DH5-α cells and selected for ampicillin resistance. Colonies were amplified, and plasmid DNA was purified and sequenced using primers complementary to the pSuppressor-Adeno vector to verify the integrity of the shRNA construct. Shuttle plasmids containing siDELTA and small interfering green fluorescent protein (siGFP) (Imgenex) inserts were linearized using *Pac*1 digestion and cotransfected with linearized pacAd5 plasmid DNA into human embryonic kidney (HEK)-293 cells using Ca^{2+}-phosphate precipitation. After 10 days and the appearance of viral foci, cells and media were harvested, freeze-thawed three times, and centrifuged, and the supernatant was collected for viral amplification.

Lysates from HEK-293 cells infected with primary viral isolates for 36 h were collected, and the amplified virus was purified by performing CsCl centrifugation. The viral band was purified on a P-10 column (Amersham, Piscataway, NJ) eluted with 2.0 ml of viral preservation medium. To determine the particle-to-infectious unit (PFU) ratio, the virus was serially diluted and applied to HEK-293 cells. After a 3-day infection, viable cells were determined by the incorporation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO). The calculated PFU for each purified virus was 1.9×10^{10} for siDELTA and 2.5×10^{10} for siGFP. The purified virus was stored in 10-μl aliquots at −80°C.

Adenoviral infection. VSM cells at ~70% confluence were infected with purified adenovirus (Ad)/CaMKII siRNA or Ad/GFP siRNA in the presence of 10% FBS medium. After infection (72–96 h), cells were used for experiments. In other experiments, VSM cells at 70% confluence were infected using Ad/Flag-tagged FRNK. The same multiplicity of infection (MOI) of Ad/β-galactosidase (Ad/β-gal) was used for virus controls. After infection (48 h) in the presence of 10% FBS medium, cells were used for experiments. Ad/Flag-tagged FRNK and Ad/β-gal were provided by Dr. Rebecca S. Keller (Albany Medical College, Albany, NY).

Adhesion assay. VSM cells grown in 10% FBS medium were used in the experiments. At confluence, cells were trypsinized and suspended in 0.4% FBS medium for 30 min. Either suspended cells ($n = 500,000$) in 0.5 ml of 0.4% FBS medium were replated onto fibronectin (Sigma)-coated 24-well dishes (10 μg/ml), or 100,000 suspended cells in 1 ml of 0.4% FBS medium were plated into six wells precoated with poly-L-lysine (Becton Dickinson, Bedford, MA). After defined periods of time, the medium was removed and 1× SDS sample buffer (10% glycerol, 1% SDS, 100 mM Tris, 5% 2-mercaptoethanol, and 0.1% bromphenol blue, pH 7.6) was added to the wells containing the adhering cells, and cells were collected using a cell scraper. The same number of suspended cells were used as a negative control. To test adhesion-dependent CaMKII activation and autophosphorylation on Thr[287], both suspended and adhering cells were treated with the Ca^{2+} ionophore ionomycin (Calbiochem, La Jolla, CA) at 0.5 μM concentration for 30 s as a positive control to determine the maximal activation responses. To collect protein in suspended cells, equal volumes of 20% ice-cold trichloroacetic acid were added, incubated for 30 min at 4°C and then centrifuged at 14,000 g at 4°C for 1 min. The pellet was washed with acetone three times, dried in the air, and then dissolved in 100 μl of 1× SDS sample buffer and sonicated at 22°C for 30 min.

Western blot analysis. Samples were heated at 95°C for 5 min and centrifuged at 14,000 g for 2 min at 22°C. Proteins were resolved by SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. Blots were blocked in 5% nonfat milk-Tris-buffered saline with Tween 20 (TBST) for 30 min at 22°C, incubated with recommended dilutions of primary antibody in TBST for 1 h at 22°C, and washed three times with TBST. The blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody in TBST for 1 h at 22°C and washed three times with TBST before detection using a commercial chemiluminescence kit (Amersham) and exposure to X-ray film.

Antibodies. Anti-phospho-CaMKII (Thr[287]) and anti-CaMKII δ₂-isoform antibodies were produced as previously described (29, 34). Other antibodies were purchased from commercial sources as follows: anti-phospho-ERK1/2 antibody (Cell Signaling, Beverly, MA), anti-phospho-FAK (Tyr[397]) antibody (Upstate Biotechnology, Lake Placid, NY), anti-phospho-paxillin (Tyr[118]) antibody (Cell Signaling),

anti-ERK2 antibody (Cell Signaling), anti-FAK polyclonal antibody (Upstate Biotechnology), and anti-β-actin antibody (Sigma).

Statistics. Values shown are means ± SE. Statistically significant differences in mean values were judged using Student's *t*-test (two-tailed, paired comparison). $P < 0.05$ represents a statistical difference between two groups.

RESULTS

CaMKII is activated upon VSM cell adhesion. Previous studies indicated that in VSM cells, CaMKII functions as an intermediate for Ca^{2+}-induced ERK1/2 activation through transactivation of EGFRs (1, 10, 11). In those studies, a GPCR agonist or the Ca^{2+} ionophore ionomycin was used to stimulate increases in free intracellular Ca^{2+} and activation of CaMKII. Adhesion of VSM cells to multiple substrates has also been reported to stimulate intracellular Ca^{2+} transients (28), and adhesion-dependent activation of ERK1/2 has been described in a number of systems (7, 20, 37). To determine whether CaMKII was activated in response to VSM cell adhesion, cultured cells were enzymatically dispersed and replated on fibronectin (10 µg/ml)-coated plates after 30-min suspension in 0.4% FBS medium. CaMKII activation was assessed by performing Western blot analysis using a specific antibody for the kinase autophosphorylated on Thr[287] (34). Autophosphorylation at this site is dependent on activation of subunits by CaM (25). CaMKII autophosphorylation was stimulated within 25 min after cell adhesion to fibronectin compared with unstimulated suspended cells, although the addition of ionomycin further increased activation levels (Fig. 1*A*). Total CaMKII protein in adhering cells after 25 min was similar to the starting level of protein in suspended cells, suggesting that most of the cells had adhered by this time. Addition of 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN-93) (30 µM), a selective inhibitor of CaMKII activation, attenuated adhesion-dependent autophosphorylation of CaMKII as expected (data not shown).

To assess CaMKII activation at early time points after plating but before adhesion was complete, autophosphorylation signals were normalized against recovered CaMKII protein and then compared with the normalized signal from unstimulated suspended cells. CaMKII activation was maximally increased fourfold by ionomycin stimulation in both suspended and adhering cells (Fig. 1*B*). CaMKII activation in response to adhesion alone was ~75% of maximal ionomycin-induced activation, or about threefold that of control levels. Using this analysis, CaMKII activation could be detected in VSM cells as early as 1 min after being replated on fibronectin-coated dishes.

In addition to fibronectin, CaMKII activation was observed to occur rapidly upon cell adhesion to multiple substrates, including collagen, laminin, and fibrinogen (data not shown). However, the addition of solubilized fibronectin to suspended cells failed to activate CaMKII (Fig. 1*C*), suggesting that integrin binding alone was insufficient to trigger the response.

Adhesion-dependent CaMKII activation is independent of integrin signaling. Activation of FAK by tyrosine phosphorylation after integrin engagement causes recruitment of adhesion molecules such as paxillin, talin, and vinculin to form a focal adhesion complex (20, 37). To determine whether the signal for CaMKII activation was dependent on FAK activation, FRNK, an endogenous inhibitor of FAK (27, 32), was over-
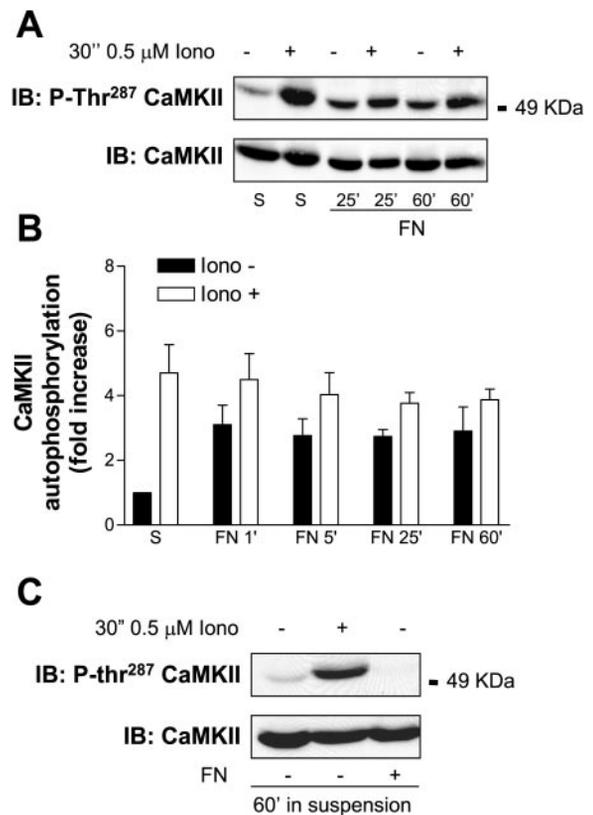

Fig. 1. CaMKII is activated upon cell adhesion to fibronectin (FN). Vascular smooth muscle (VSM) cells were plated onto fibronectin-coated dishes at different time points (1, 5, 25, and 60 min). The same amount of suspended cells (S) was used as a negative control. Either suspended cells or adhering cells were treated with the Ca^{2+} ionophore ionomycin (Iono) for 30 s and used as positive controls to determine maximal CaMKII activation responses. *A, top*: immunoblot (IB) with anti-phospho (P)-Thr[287] CaMKII antibody to assess autophosphorylated CaMKII; *bottom*: immunoblot with anti-CaMKII antibody used to detect total CaMKII protein. Blots are representative of 3 independent experiments. *B*: phospho-Thr[287] CaMKII blots were normalized to total CaMKII blots, and changes are expressed relative to data in unstimulated suspended cells. Solid bars represent cells without Iono treatment, and open bars represent cells after Iono treatment. Values are means ± SE; $n = 3$. *C, top*: immunoblot with anti-phospho-Thr[287] CaMKII antibody; *bottom*: immunoblot with anti-CaMKII antibody. VSM cells were treated with or without 10 µg/ml soluble FN while in suspension. Suspended cells were also stimulated with Iono for 30 s and used as a positive control. Blots shown are representative of 3 independent experiments.

expressed in VSM cells. FRNK is a COOH-terminal fragment of FAK that inhibits phosphorylation of FAK on Tyr[397] and interferes with FAK protein-protein interactions (26). As expected, adhesion-dependent tyrosine phosphorylation and activation of FAK and paxillin was inhibited by the overexpression of FRNK introduced by adenoviral infection (Fig. 2*A*). In contrast, under these conditions of FRNK overexpression, adhesion-dependent activation of CaMKII was unaffected (Fig. 2*B*), indicating that CaMKII activation is not dependent on prior activation of FAK.

To determine whether adhesion-dependent CaMKII activation was dependent on integrin signaling by pathways other than FAK activation, cells were plated onto poly-L-lysine-coated dishes. While poly-L-lysine promotes VSM cell adhesion, the cell-substrate interactions are thought to be based on charge interactions and the substrate is commonly used as a
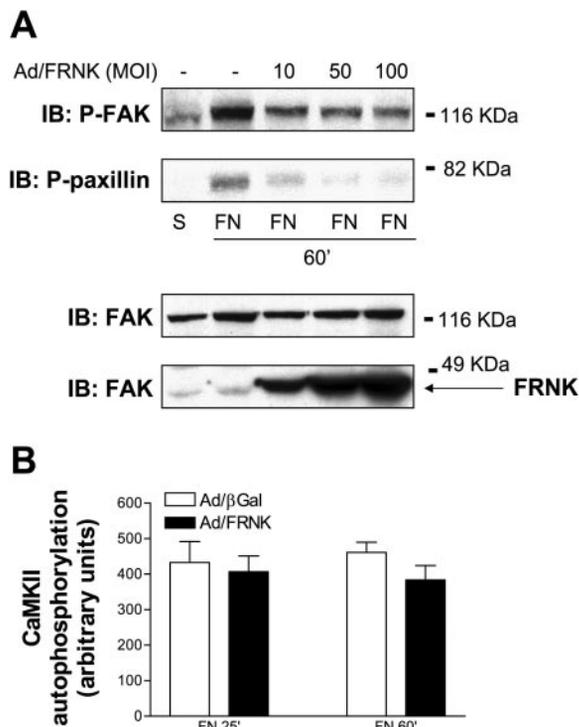
Fig. 2. *A*: Inhibition of adhesion-dependent focal adhesion kinase (FAK) and paxillin activation by FAK-related nonkinase (FRNK). VSM cells were infected with either 50 multiplicities of infection (MOI) of adenovirus (Ad)/β-galactosidase (Ad/β-gal) or Ad/FRNK at 10, 50 and 100 MOI. After infection (48 h), cells were trypsinized and plated onto FN-coated dishes for 60 min. The same number of suspended cells was used as a negative control. Samples were analyzed by immunoblotting using antibodies against FAK-phosphorylated at Tyr^{397} (IB: P-FAK) and paxillin phosphorylated at Tyr^{118} (IB: P-paxillin). FRNK expression was detected using an anti-FAK polyclonal antibody (IB: FAK). Total FAK protein was used as a loading control. Blots shown are representative of 3 independent experiments. *B*: adhesion-dependent activation of CaMKII is independent of FAK activation. VSM cells were infected with either Ad/β-Gal or Ad/FRNK at 50 MOI. After infection (48 h), cells were trypsinized and plated onto FN-coated dishes for 25 and 60 min. The same numbers of suspended cells were used as a negative control. Data were quantified by performing densitometric scanning from the immunoblots with anti-phospho-Thr^{287} CaMKII antibody, and means $\pm$ SE were plotted; $n = 3$.

negative control for integrin-dependent adhesion and signaling (21). Although FAK and paxillin were slowly activated after adhesion to poly-L-lysine, CaMKII was rapidly and robustly activated, temporally preceding FAK activation (Fig. 3). Similar to the results in cells adhering to fibronectin, FRNK overexpression did not inhibit activation of CaMKII in cells plated onto poly-L-lysine-coated dishes (data not shown). Taken together, these data strongly suggest that CaMKII activation upon cell adhesion is mediated by an integrin-independent pathway.

CaMKII promotes adhesion-dependent ERK activation. A number of previous studies have documented adhesion-dependent activation of ERK1/2 and implicated this response in the modulation of focal adhesion dynamics (35). Consistent with these earlier reports, ERK1/2 activation was found to be progressively activated upon VSM cell adhesion to fibronectin (Fig. 4*A*). FRNK overexpression blocked activation of ERK1/2 in cells plated onto fibronectin-coated dishes, indicating a key role for FAK activation in that process (Fig. 4).

To determine whether CaMKII mediated or modulated adhesion-dependent activation of ERK1/2, both molecular and
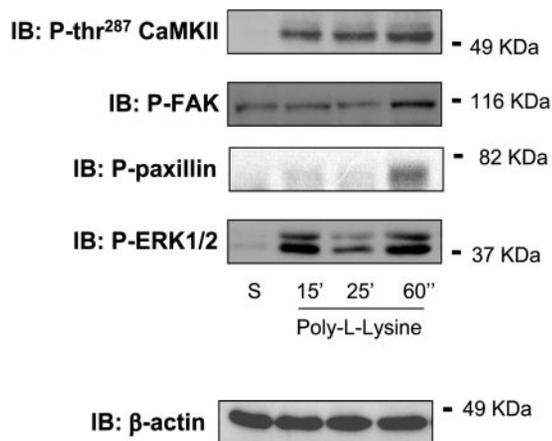

Fig. 3. Adhesion-dependent CaMKII activation on poly-L-lysine-coated dishes. VSM cells were plated onto poly-L-lysine-coated plates for 15, 25, and 60 min. The same number of suspended cells was used as a negative control. Immunoblots with anti-phospho-Thr^{287} CaMKII, anti-phospho-Tyr^{397} FAK, anti-phospho-Tyr^{118} paxillin, anti-phospho-ERK1/2, and anti-β-actin (loading control) antibodies are shown. Blots are representative of 3 independent experiments.

pharmacological approaches were used to suppress CaMKII. In the molecular approach, siRNA (6) was used to suppress CaMKII-δ expression, the principal CaMKII gene product expressed in these cells (29). SiRNA target sequences were identified and characterized in preliminary experiments with
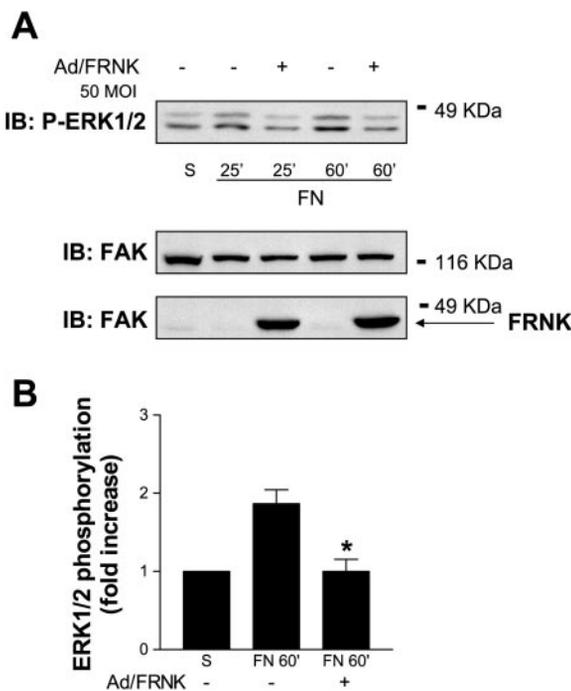

Fig. 4. Inhibition of adhesion-dependent ERK activation by FRNK. VSM cells were infected with either Ad/β-gal or Ad/FRNK at 50 MOI. After infection (48 h), cells were trypsinized and plated onto FN-coated dishes for 25 and 60 min. The same number of suspended cells was used as a negative control. *A*, *top*: immunoblot with anti-phospho-ERK1/2 antibody; *bottom*: immunoblot with anti-FAK polyclonal antibody to detect FRNK expression and total FAK protein, which was used as a loading control. Blots shown are representative of 3 independent experiments. *B*: quantified data from phospho-ERK1/2 blots in *A* shown as means $\pm$ SE; $n = 3$. *$P < 0.05$, statistical difference between FN 60 min and FN 60 min $+$ Ad/FRNK.

the use of transient transfection approaches. Two candidate sequences were then cloned as shRNA and introduced into VSM cells using adenoviral vectors (39). Infection of VSM cells with adenovirus containing shRNA targeting the CaMKII-δ gene (Ad/siDELTA) resulted in dose (i.e., MOI)-dependent decreases in CaMKII-δ protein expression levels compared with cells infected with control adenovirus targeting GFP (data not shown). The addition of 150 MOI of Ad/siDELTA resulted in a $59 \pm 4\%$ ($n = 11$) decrease in CaMKII-δ protein (Fig. 5A). ERK1/2 activation in response to the addition of iomomycin was inhibited in cells with reduced levels of CaMKII, while phorbol 12,13-dibutyrate (PDBu)-stimulated ERK1/2 activation was unaffected (Fig. 5, A and B). Similar results were obtained after CaMKII-δ gene silencing using an alternative target sequence directed against the COOH terminus of CaMKII-δ_2 (data not shown). Together these data suggest that Ad/siDELTA infection specifically affects Ca^{2+}- and CaMKII-dependent, but not PKC-dependent, pathways, resulting in ERK1/2 activation.

After suppression of CaMKII-δ expression with 150 MOI Ad/siDELTA, VSM cells were suspended and replated onto fibronectin-coated dishes to assess adhesion-dependent events.
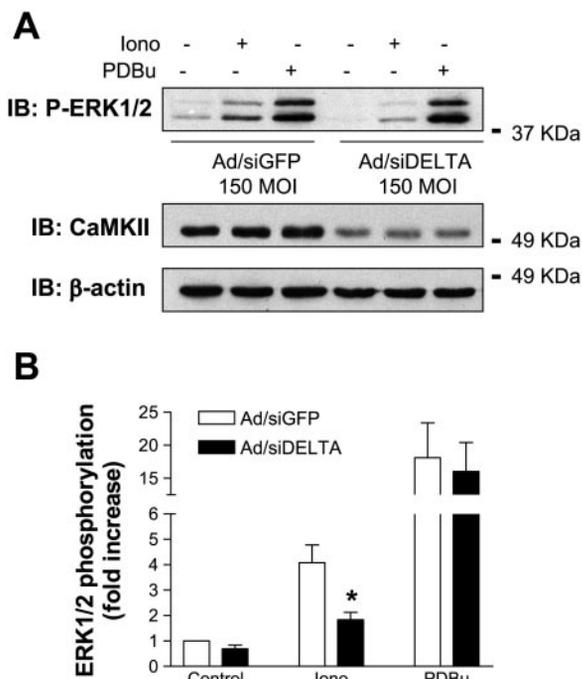

Fig. 5. Inhibition of ionomycin stimulated ERK activation by CaMKII-δ gene silencing. A: VSM cells were infected with adenoviral small interfering green fluorescent protein (Ad/siGFP) or Ad/siDELTA (adenoviral sequence targeting all CaMKII δ-isoforms) at 150 MOI for 72 h and then stimulated with either 0.5 μM ionomycin (Iono) for 5 min or 0.5 μM phorbol 12,13-dibutyrate (PDBu) for 10 min. *Top*: immunoblot in which anti-phospho-ERK1/2 antibody was used to assess ERK activation; *middle*: immunoblot with anti-CaMKII antibody used to assess protein levels after introduction of siDELTA; *bottom*: immunoblot in which anti-β-actin was used to evaluate gel loading. Blots shown are representative of 9 independent experiments. B: densitometric quantification of phospho-ERK1/2 in cells treated as described in A. Values are normalized to phospho-ERK1/2 levels from unstimulated cells infected with Ad/siGFP. Open bars represent cells infected with Ad/siGFP, and solid bars represent cells infected with Ad/siDELTA. Data shown are means ± SE; $n = 9$. *$P < 0.05$, statistical difference between siGFP- and siDELTA-infected cells after ionomycin treatment.
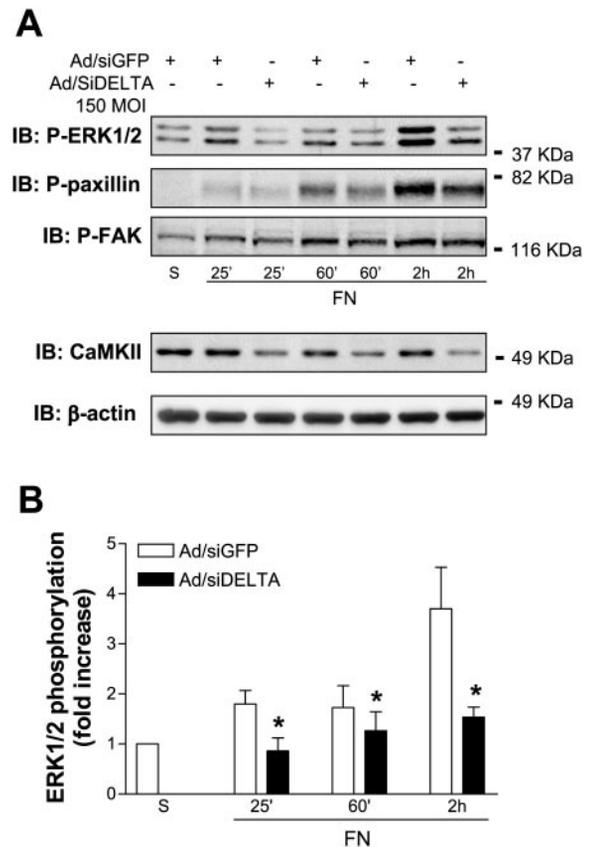

Fig. 6. Inhibition of adhesion-dependent ERK and paxillin activation by CaMKII-δ gene silencing. VSM cells were infected with either Ad/siGFP or Ad/siDELTA at 150 MOI 72 h after infection, trypsinized, and replated after 30 min on fibronectin-coated plates for 25 min, 60 min, and 2 h. The same numbers of suspended cells were used as a negative control. A: immunoblots with anti-phospho-ERK1/2, anti-phospho-Tyr[118] paxillin, anti-phospho-Tyr[397] FAK, anti-CaMKII, and anti-β-actin (loading control) antibodies are shown. Blots shown are representative of 4 independent experiments. B: densitometric scans from phospho-ERK1/2 blots in A normalized to unstimulated suspended cells. Values shown are means ± SE; $n = 4$. Open bars represent control cells infected with Ad/siGFP, and solid bars represent cells infected with Ad/siDELTA. *$P < 0.05$, statistical difference between siGFP- and siDELTA-infected cells at each time point.

Adhesion-dependent increases in ERK1/2 activation between 25 min and 2 h were significantly inhibited after suppression of CaMKII-δ expression (Fig. 6, A and B). As an alternative approach, addition of KN-93, which selectively inhibits CaM-dependent activation of CaMKII (1), inhibited adhesion-dependent activation of ERK1/2 (Fig. 7A). These data indicate a positive role for CaMKII in modulating adhesion-dependent activation of ERK1/2.

CaMKII modulates adhesion-dependent signaling down-stream of FAK activation. Although CaMKII activation is not dependent on prior activation of FAK (Fig. 4), it facilitated adhesion-dependent signaling, leading to ERK1/2 activation (Figs. 6 and 7). To determine whether CaMKII promotes ERK activation through modulation of FAK, adhesion-dependent FAK activation was assessed in cells after CaMKII-δ gene silencing (Fig. 6A) or CaMKII inhibition with KN-93 (Fig. 7B). Inhibition of CaMKII using either approach had no significant effects on adhesion-stimulated FAK activation ($n = 3$). Interestingly, tyrosine phosphorylation of paxillin, a re-
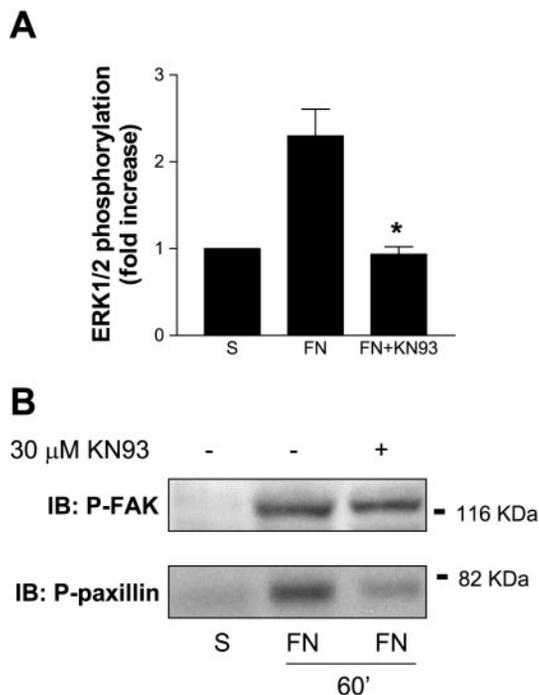
Fig. 7. Inhibition of adhesion-dependent ERK and paxillin activation using 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93). VSM cells in suspension were pretreated with or without 30 μM KN-93 for 30 min and then plated onto FN-coated plates for 60 min. *A*: quantified data from phospho-ERK1/2 blots. Values are means $\pm$ SE; $n = 3$. *$P < 0.05$, statistical difference between FN 60 min and FN 60 min + KN-93. *B*, *top*: immunoblot with anti-phospho-Tyr[397] FAK antibody; *bottom*: immunoblot with anti-phospho-Tyr[118] paxillin antibody. Blots shown are representative of 3 independent experiments.

sponse known to be regulated by the nonreceptor tyrosine kinases FAK and Src (4, 37), was consistently inhibited (31 $\pm$ 7%; $n = 3$ at 2 h) after CaMKII protein suppression with siDELTA compared with siGFP control (Fig. 6*A*) or inhibition of CaMKII activation with KN-93 (Fig. 7*B*). These data suggest that facilitation of adhesion-dependent signaling events resulting in ERK1/2 activation by CaMKII occurs at steps downstream from FAK activation but proximal to paxillin activation.

CaMKII mediates integrin-independent ERK1/2 activation. Having demonstrated that CaMKII promoted FAK-dependent ERK1/2 activation in cells plated on fibronectin (Figs. 6 and 7), we tested the role of CaMKII in mediating adhesion-stimulated ERK1/2 activation by an integrin-independent pathway in cells plated onto poly-L-lysine-coated dishes. Suppression of CaMKII expression using siDELTA inhibited ERK1/2 activation at all time points after adhesion onto poly-L-lysine-coated plates (Fig. 8). Together, these data indicate that CaMKII facilitates adhesion-dependent ERK1/2 activation by both integrin-dependent and integrin-independent mechanisms.

DISCUSSION

CaMKII-dependent activation of ERK1/2 has been described previously in VSM cells (10, 11) and other cells (16) in response to activation by GPCR agonists, depolarizing stimuli, or ionophores that result in increased free intracellular Ca^{2+} levels. Integrin-mediated cell adhesion has been reported to result in the activation and localization of ERK1/2 in newly forming focal adhesions (7). ERK1/2 activation under these conditions, or under similar conditions in the leading edge of migrating cells, is likely to be important in regulating focal adhesion dynamics (37) and cytoskeletal interactions (15). These cellular processes are important components of pathophysiological conditions such as restenosis or atherosclerosis, which involve VSM cell proliferative and migratory responses. A number of mechanisms for integrin coupling to ERK activation have been described, including FAK and Src-dependent pathways (7), EGFR transactivation (11), and caveolin/Fyn-dependent pathways (36). In the present study, ERK1/2 activation after VSM cell adhesion was found to be dependent largely on FAK activity as indicated by the inhibition of ERK activation after overexpressing the FAK inhibitor FRNK (Fig. 4). However, our studies have also revealed additional pathways facilitating adhesion-dependent ERK activation that results from the activation of the multifunctional serine/threonine kinase CaMKII.

Upon activation by CaM binding, CaMKII undergoes rapid autophosphorylation on a specific threonine residue (Thr[287] in the δ_2-subunit) that confers CaM-independent or autonomous activity to the kinase. In the present study, we assessed the phosphorylation of Thr[287] in CaMKII-δ_2, the principal isoform expressed in these cells (29), by performing Western blot analysis using a phospho-peptide-specific antibody as an indicator of CaMKII activation. Adhesion-dependent responses were compared with positive control responses in suspended cells or adhering cells maximally activated with the Ca^{2+}-ionophore ionomycin. Additional experiments (not shown) confirmed that Thr[287] phosphorylation under these conditions correlated with an increase in autonomous CaMKII activity. Using these approaches, we detected rapid increases in CaMKII activation (within 1 min) in response to the adhesion of VSM cells on fibronectin-coated plates. Similar levels of CaMKII activation upon adhesion to collagen and laminin were observed, suggesting that CaMKII activation is substrate independent.

In the present study, we found no direct evidence for CaMKII activation after the addition of soluble fibronectin to
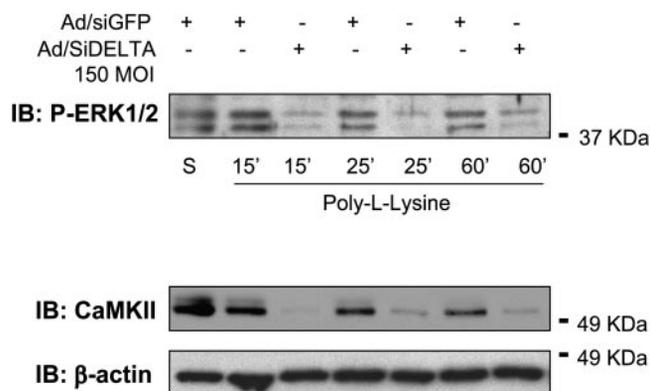

Fig. 8. Inhibition of adhesion-dependent ERK activation on poly-L-lysine using siDELTA. VSM cells were infected with either Ad/siGFP or Ad/siDELTA at 150 MOI. After 96 h, infected cells were trypsinized and plated onto poly-L-lysine-coated dishes for 15, 25, and 60 min. The same numbers of suspended cells were used as a negative control. *Top*: immunoblot with anti-phospho-ERK1/2 antibody; *middle*: immunoblot with anti-CaMKII antibody to assess protein levels after introduction of siDELTA; *bottom*: immunoblot with anti-β-actin antibody used as a loading control. Blots shown are representative of 3 independent experiments.

suspended cells. However, adhesion-dependent CaMKII activation in VSM cells was not inhibited by FRNK overexpression and therefore appears to be independent of FAK activity and focal adhesion formation per se. The results of experiments in which we used cells plated onto poly-L-lysine-coated dishes indicate that CaMKII activation after adhesion is not dependent on integrin signaling. These results, along with the rapid kinetics of CaMKII activation that precede activation of FAK and paxillin, suggest that CaMKII activation is triggered by an adhesion-dependent but integrin signaling-independent mechanism in parallel with integrin-dependent FAK activation (summarized in Fig. 9). The integrin-independent mechanisms underlying the activation of CaMKII in response to cell adhesion are not known. However, spontaneous Ca^{2+} transients in VSM cells plated onto poly-L-lysine-coated dishes have a precedent (28), and at least one potential integrin-independent pathway has been reported for Ca^{2+} release involving CD44 cell surface receptors for hyaluronan and the cytoskeletal protein ankyrin to form a CD44-ankyrin-sarcoplasmic reticulum IP_3 receptor protein complex (30).

Regardless of the precise mechanisms, the present studies indicate that adhesion-dependent activation of CaMKII facilitates FAK-dependent tyrosine phosphorylation of paxillin and ERK1/2 activation and also facilitates ERK activation by integrin- and FAK signaling-independent pathways (summarized in Fig. 9). Previous studies implicating CaMKII in the regulation of ERK1/2 signaling have relied heavily on pharmacological approaches (1, 11) or overexpression of mutant CaMKII subunits (10). In the present study, the gene silencing approach using siRNA to suppress expression of endogenous CaMKII-δ expression in VSM cells provided a strong molecular confirmation of the importance of this multifunctional kinase in regulating ERK1/2 activation by Ca^{2+}-mobilizing

stimuli and by cell adhesion. A role for CaMKII in mediating adhesion-dependent activation ERK also was recently reported in a human thyroid cell line (TAD-2 cells) and in Hep3B (14). On the basis of those previous studies and the present study using primary cultures of VSM, it seems that adhesion-dependent activation of CaMKII and subsequent ERK1/2 activation may be of importance in diverse cell types.

In the published studies using TAD-2 and Hep3B cells, activation of ERK in response to adhesion to fibronectin was proposed to occur via a mechanism involving the formation of a CaMKII/Raf-1 complex, leading to phosphorylation and activation of Raf-1 by CaMKII and subsequent ERK signaling (14). However, it was not clearly determined in those studies whether CaMKII directly interacted with and regulated Raf-1 activity or whether the interaction and regulation were indirect and involved intermediary proteins. In the present study, we have shown that gene silencing of CaMKII-δ or CaMKII inhibition with KN-93 inhibited adhesion-dependent paxillin tyrosine phosphorylation and ERK1/2 activation. Because FAK autophosphorylation on Tyr^{397} was unaffected by these treatments and paxillin tyrosine phosphorylation is FAK and Src dependent, we propose that CaMKII acts by facilitating the recruitment of focal adhesion molecules after FAK activation. Tyrosine-phosphorylated paxillin may promote ERK activation by scaffolding proximal elements in the cascade such as Raf and MEK (4). Such a mechanism would be consistent with CaMKII-dependent Raf activation and CaMKII-Raf protein interactions (14) via the formation of a larger signaling complex. Our experiments using poly-L-lysine as a substrate suggest that in addition to facilitating FAK-dependent paxillin and ERK1/2 activation, CaMKII may also promote ERK1/2 activation via an integrin-independent pathway. Assessment of the relative contribution of this mode of adhesion-dependent ERK1/2 activation in cells adhering to various substrates requires further studies.

FAK, paxillin, and ERK have all been shown to be critical in the regulation of focal adhesion dynamics in migrating cells (37). A number of studies have also indicated that CaMKII modulates VSM cell migration, demonstrated by the changes in cell migration with the use of pharmacological inhibitors of CaMKII, KN-93 and 1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), respectively (18, 23), or by overexpressing CaMKII-$δ_2$ mutants (constitutively active and kinase negative) to manipulate CaMKII activities (24). At the cellular level, CaMKII-$δ_2$ has been found to be enriched in the pseudopodia of migrating cells (17). Additional studies, under conditions in which cells that are stimulated to migrate are required to evaluate more carefully a possible role for CaMKII in regulating VSM cell focal adhesion dynamics.

In summary, we have demonstrated that in VSM cells, CaMKII is activated in response to cell adhesion but that, in contrast to many other signaling molecules, this response is rapid and does not require integrin-dependent FAK activation. One function of adhesion-dependent CaMKII activation in VSM cells is to modulate FAK-dependent and FAK-independent ERK1/2 activation. On the basis of previous studies documenting the importance of ERK1/2 in regulating focal adhesion dynamics, the present results provide insight into potential mechanisms by which this multifunctional serine/threonine kinase could modulate VSM cell migration.
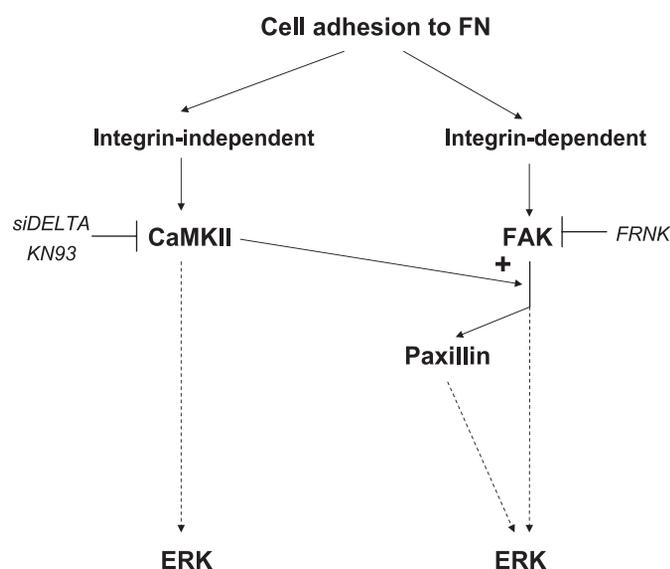

Fig. 9. Proposed model for modulation of adhesion-dependent ERK1/2 activation by CaMKII. CaMKII is rapidly activated upon VSM cell adhesion by an integrin signaling-independent mechanism, because CaMKII activation preceded FAK activation, was unaffected by FAK inhibition with FRNK, and occurred after integrin-independent adhesion to a poly-L-lysine-coated dish. Activation of CaMKII regulates ERK1/2 signaling through both FAK-dependent and FAK-independent mechanisms. Regulation of FAK-dependent tyrosine phosphorylation of paxillin and ERK1/2 activation by CaMKII occurs at a step distal to FAK autophosphorylation and activation.

REFERENCES

1. **Abraham ST, Benscoter HA, Schworer CM, and Singer HA.** A role for Ca^{2+}/calmodulin-dependent protein kinase II in the mitogen-activated protein kinase signaling cascade of cultured rat aortic vascular smooth muscle cells. *Circ Res* 81: 575–584, 1997.

2. **Bilato C, Curto KA, Monticone RE, Pauly RR, White AJ, and Crow MT.** 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. *J Clin Invest* 100: 693–704, 1997.

3. **Bouvard D, Molla A, and Block MR.** Calcium/calmodulin-dependent protein kinase II controls $\alpha_5\beta_1$ integrin-mediated inside-out signaling. *J Cell Sci* 111: 657–665, 1998.

4. **Brown MC and Turner CE.** Paxillin: adapting to change. *Physiol Rev* 84: 1315–1339, 2004.

5. **Chen Q, Kinch MS, Lin TH, Burridge K, and Juliano RL.** Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J Biol Chem* 269: 26602–26605, 1994.

6. **Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T.** Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494–498, 2001.

7. **Fincham VJ, James M, Frame MC, and Winder SJ.** Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. *EMBO J* 19: 2911–2923, 2000.

8. **Geisterfer AA, Peach MJ, and Owens GK.** Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ Res* 62: 749–756, 1988.

9. **Giannone G, Rondé P, Gaire M, Haiech J, and Takeda K.** Calcium oscillations trigger focal adhesion disassembly in human U87 astrocytoma cells. *J Biol Chem* 277: 26364–26371, 2002.

10. **Ginnan R, Pfleiderer PJ, Pumiglia K, and Singer HA.** PKC-δ and CaMKII-δ_2 mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle. *Am J Physiol Cell Physiol* 286: C1281–C1289, 2004.

11. **Ginnan R and Singer HA.** CaM kinase II-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle. *Am J Physiol Cell Physiol* 282: C754–C761, 2002.

12. **Hagel M, George EL, Kim A, Tamimi R, Opitz SL, Turner CE, Imamoto A, and Thomas SM.** The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol* 22: 901–915, 2002.

13. **Hynes RO.** Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69: 11–25, 1992.

14. **Illario M, Cavallo AL, Bayer KU, Di Matola T, Fenzi G, Rossi G, and Vitale M.** Calcium/calmodulin-dependent protein kinase II binds to Raf-1 and modulates integrin-stimulated ERK activation. *J Biol Chem* 278: 45101–45108, 2003.

15. **Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, and Cheresh DA.** Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol* 137: 481–492, 1997.

16. **Kutcher LW, Beauman SR, Gruenstein EI, Kaetzel MA, and Dedman JR.** Nuclear CaMKII inhibits neuronal differentiation of PC12 cells without affecting MAPK or CREB activation. *Am J Physiol Cell Physiol* 284: C1334–C1345, 2003.

17. **Lin YH, Park ZY, Lin D, Brahmbhatt AA, Rio MC, Yates JR III, and Klemke RL.** Regulation of cell migration and survival by focal adhesion targeting of Lasp-1. *J Cell Biol* 165: 421–432, 2004.

18. **Lundberg MS, Curto KA, Bilato C, Monticone RE, and Crow MT.** Regulation of vascular smooth muscle migration by mitogen-activated protein kinase and calcium/calmodulin-dependent protein kinase II signaling pathways. *J Mol Cell Cardiol* 30: 2377–2389, 1998.

19. **McNamara CA, Sarembock IJ, Bachhuber BG, Stouffer GA, Ragosta M, Barry W, Gimple LW, Powers ER, and Owens GK.** Thrombin and vascular smooth muscle cell proliferation: implications for atherosclerosis and restenosis. *Semin Thromb Hemost* 22: 139–144, 1996.

20. **Miranti CK and Brugge JS.** Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol* 4: E83–E90, 2002.

21. **Nojima Y, Morino N, Mimura T, Hamasaki K, Furuya H, Sakai R, Sato T, Tachibana K, Morimoto C, and Yazaki Y.** Integrin-mediated cell adhesion promotes tyrosine phosphorylation of p130Cas, a Src homology 3-containing molecule having multiple Src homology 2-binding motifs. *J Biol Chem* 270: 15398–15402, 1995.

22. **Panetti TS.** Tyrosine phosphorylation of paxillin, FAK, and p130Cas: effects on cell spreading and migration. *Front Biosci* 7: d143–d150, 2002.

23. **Pauly RR, Bilato C, Sollott SJ, Monticone R, Kelly PT, Lakatta EG, and Crow MT.** Role of calcium/calmodulin-dependent protein kinase II in the regulation of vascular smooth muscle cell migration. *Circulation* 91: 1107–1115, 1995.

24. **Pfleiderer PJ, Lu KK, Crow MT, Keller RS, and Singer HA.** Modulation of vascular smooth muscle cell migration by calcium/calmodulin-dependent protein kinase II-δ_2. *Am J Physiol Cell Physiol* 286: C1238–C1245, 2004.

25. **Rich RC and Schulman H.** Substrate-directed function of calmodulin in autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II. *J Biol Chem* 273: 28424–28429, 1998.

26. **Richardson A and Parsons T.** A mechanism for regulation of the adhesion-associated protein tyrosine kinase pp125[FAK]. *Nature* 380: 538–540, 1996.

27. **Schaller MD, Borgman CA, and Parsons JT.** Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125[FAK]. *Mol Cell Biol* 13: 785–791, 1993.

28. **Scherberich A, Campos-Toimil M, Rondé P, Takeda K, and Beretz A.** Migration of human vascular smooth muscle cells involves serum-dependent repeated cytosolic calcium transients. *J Cell Sci* 113: 653–662, 2000.

29. **Schworer CM, Rothblum LI, Thekkumkara TJ, and Singer HA.** Identification of novel isoforms of the delta subunit of Ca^{2+}/calmodulin-dependent protein kinase II: differential expression in rat brain and aorta. *J Biol Chem* 268: 14443–14449, 1993.

30. **Singleton PA and Bourguignon LYW.** CD44 interaction with ankyrin and IP_3 receptor in lipid rafts promotes hyaluronan-mediated Ca^{2+} signaling leading to nitric oxide production and endothelial cell adhesion and proliferation. *Exp Cell Res* 295: 102–118, 2004.

31. **Sjaastad MD, Lewis RS, and Nelson WJ.** Mechanisms of integrin-mediated calcium signaling in MDCK cells: regulation of adhesion by IP_3- and store-independent calcium influx. *Mol Biol Cell* 7: 1025–1041, 1996.

32. **Taylor JM, Mack CP, Nolan K, Regan CP, Owens GK, and Parsons JT.** Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells. *Mol Cell Biol* 21: 1565–1572, 2001.

33. **Tran POT, Hinman LE, Unger GM, and Sammak PJ.** A wound-induced $[Ca^{2+}]_i$ increase and its transcriptional activation of immediate early genes is important in the regulation of motility. *Exp Cell Res* 246: 319–326, 1999.

34. **Van Riper DA, Schworer CM, and Singer HA.** Ca^{2+}-induced redistribution of Ca^{2+}/calmodulin-dependent protein kinase II associated with an endoplasmic reticulum stress response in vascular smooth muscle. *Mol Cell Biochem* 213: 83–92, 2000.

35. **Volmat V and Pouysségur J.** Spatiotemporal regulation of the p42/p44 MAPK pathway. *Biol Cell* 93: 71–79, 2001.

36. **Wary KK, Mariotti A, Zurzolo C, and Giancotti FG.** A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell* 94: 625–634, 1998.

37. **Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, and Horwitz AF.** FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol* 6: 154–161, 2004.

38. **Wu X, Mogford JE, Platts SH, Davis GE, Meininger GA, and Davis MJ.** Modulation of calcium current in arteriolar smooth muscle by $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin ligands. *J Cell Biol* 143: 241–252, 1998.

39. **Xia H, Mao Q, Paulson HL, and Davidson BL.** siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* 20: 1006–1010, 2002.

40. **Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, Miyazaki H, and Iwao H.** Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. *Arterioscler Thromb Vasc Biol* 23: 795–801, 2003.