Modulation of vascular smooth muscle cell migration by calcium/calmodulin-dependent protein kinase II-δ2

Paul J. Pfeiderer, Katherine Kun Lu, Michael T. Crow, Rebecca S. Keller, and Harold A. Singer. Modulation of vascular smooth muscle cell migration by calcium/calmodulin-dependent protein kinase II-δ2. Am J Physiol Cell Physiol 286: C1238–C1245, 2004. First published February 4, 2004; 10.1152/ajpcell.00536.2003.—Previous studies demonstrated a requirement for multifunctional Ca2+/calmodulin-dependent protein kinase II (CaMKII) in PDGF-stimulated vascular smooth muscle (VSM) cell migration. In the present study, molecular approaches were used specifically to assess the role of the predominant CaMKII isoform (δ2 or δc) on VSM cell migration. Kinase-negative (K43A) and constitutively active (T287D) mutant forms of CaMKIIδ2 were expressed using recombinant adenoviruses. CaMKII activities were evaluated in vitro by using a peptide substrate and in intact cells by assessing the phosphorylation of overexpressed phosphorylamban on Thr57, a CaMKII-selective phosphorylation site. Expression of kinase-negative CaMKIIδ2 inhibited substrate phosphorylation both in vitro and in the intact cell, indicating a dominant-negative function with respect to exogenous substrate. However, overexpression of the kinase-negative mutant failed to inhibit endogenous CaMKIIδ2 autophosphorylation on Thr287 after activation of cells with ionomycin, and in fact, these subunits served as a substrate for the endogenous kinase. Constitutively active CaMKIIδ2 phosphorylated substrate in vitro without added Ca2+/calmodulin and in the intact cell without added Ca2+/dependent stimuli, but it inhibited autophosphorylation of endogenous CaMKIIδ2 on Thr287. Basal and PDGF-stimulated cell migration was significantly enhanced in cells expressing kinase-negative CaMKIIδ2, an effect opposite that of KN-93, a chemical inhibitor of CaMKII activation. Expression of the constitutively active CaMKIIδ2 mutant inhibited PDGF-stimulated cell migration. These studies point to a role for the CaMKIIδ2 isoform in regulating VSM cell migration. An inclusive interpretation of results using both pharmacological and molecular approaches raises the hypothesis that CaMKIIδ2 autophosphorylation may play an important role in PDGF-stimulated VSM cell migration.

VASCULAR SMOOTH MUSCLE (VSM) cells in the medial walls of blood vessels are normally quiescent and express a differentiated phenotype that serves to generate and maintain vascular tone (18). However, vascular injury may result in dedifferentiation, migration across the internal elastic lamina, and proliferation of VSM cells to form a neointima (18, 33). Although the overall characterization of VSM within the neointima is well documented, the molecular mechanisms and signaling pathways that control VSM cell migration are not well understood.

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pressed, along with minor levels of \( \delta \) (\( \delta_h \)) splice variant that is major CaMKII product expressed, along with minor levels of \( \delta \) (\( \delta_h \)) splice variant detectable by RT-PCR and Western blot analysis. In the present study, we inhibited endogenous CaMKII2 function by using an adenoviral infection approach to introduce a kinase-negative mutant of CaMKII2 that was demonstrated to act as a dominant negative with respect to CaMKII activity. Whereas KN-93 was shown to inhibit PDGF-stimulated VSM cell migration, over-expression of the kinase-negative CaMKII2 potentiated PDGF-induced migration. Furthermore, expression of a constitutively active \( \delta_2 \)-isoform inhibited VSM cell migration. The results of these studies necessitate a careful reexamination of the mechanisms by which CaMKII regulates VSM cell migration, cognizant of potential isoform-specific functions and/or functions uniquely related to its autophosphorylation state.

MATERIALS AND METHODS

Antibodies and other materials. Production and specificity of the antibodies used for detection of the \( \delta \)-specific isoform of CaMKII and the Thr\(^{287} \) phosphorylated form of CaMKII2 were described previously (20, 31). Polyclonal antibody specific for the hemagglutinin (HA) epitope was purchased from Upstate Biotechnology (Lake Placid, NY) and monoclonal antibody specific for the Flag epitope was obtained from Sigma (St. Louis, MO). Antibody specifically recognizing the phosphorylated form of phospholamban (PLB; PT17), was purchased from Calbiochem (La Jolla, CA). PDGF-BB and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise specified. Ionomycin was purchased from Calbiochem (La Jolla, CA). PDGF-BB and all other chemicals were purchased from Sigma.

Cell culture. VSM cells were dispersed from thoracic aortas of 200- to 300-g Sprague-Dawley rats as previously described (7). Cells were cultured in combined DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) at 37°C and 5% CO\(_2\). Cells from passages 3–10 were used for experiments. Cells were grown to 70% confluence before infection with adenoviral constructs.

Cloning and adenovirus generation. Mutations of CaMKII2 were engineered using the Transformer Site Directed Mutagenesis kit (Clontech, Palo Alto, CA). Kinase-negative CaMKII2 was generated by replacing Lys\(^{43} \) with an alanine. Amino acid substitution of Thr\(^{287} \) to aspartic acid generated a constitutively active CaMKII2 mutant. M. T. Crow provided adenoviral stocks encoding kinase-negative (Ad-KN;\( \delta_2 \)) and constitutively active (Ad-CA;\( \delta_2 \)) CaMKII2. Adenovirus encoding β-galactosidase (Ad-βGal) was a gift from R. S. Keller. RT-PCR was used (Titan one-tube RT-PCR kit; Roche, Indianapolis, IN) to clone PLB from isolated rat brain RNA. Primers were designed for the hemagglutinin (HA) epitope before infection with adenoviral constructs.

Cell lysates, immunoprecipitation, and immunoblotting. Thirty minutes before experimentation, growth medium was removed from VSM cells and replaced with Hanks’ balanced salt solution (HBSS) supplemented with Ca\(^{2+}\) and 10 mM HEPES, pH 7.4. Reactions were stopped by removing HBSS, cells were lysed on ice and adding RIPA lysis buffer (4°C, 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na\(_2\)PO\(_4\), 2 mM NaVO\(_4\), 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM diethothenoi, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin) at 0.3 ml/60-mm dish. The lysates and cells were collected into 1.5-ml tubes and then centrifuged at 14,000 rpm at 4°C for 10 min, and the supernatant was collected for Western blot analysis or storage at −20°C.

For immunoprecipitation of PLB, 60-mm dishes infected and treated with an appropriate stimulus were lysed in 500 μl of immunoprecipitate (IP) buffer (500 mM Tris, pH 7.4, 50 mM NaF, 0.1 mM NaVO\(_4\), and 0.5% NP-40). Dishes were scraped, and lysates were cleared by centrifugation at 14,000 rpm at 4°C for 10 min. Monoclonal α-Flag antibody (2 μg of 40 μl of protein A beads were added to each IP (Pierce, Richmond, CA). After immunoprecipitation overnight at 4°C, they were washed three times the next day in lysis buffer followed by the addition of SDS sample buffer.

Lysates and IPs were resolved by 9 or 15% SDS-PAGE gels and transferred to nitrocellulose. The membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 (TBST) and 5% nonfat dry milk. After blocking, the membranes were incubated in primary antibody for 1 h at room temperature, washed three times with TBST, and incubated with horseradish peroxidase-conjugated secondary antibody (1:1,500 dilution; Amersham) for an additional hour at room temperature, then washed three times with TBST. Membranes were developed using chemiluminescence substrate (Amersham) and exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ). PLB phosphorylation was quantified by densitometric analysis of the immunoblot enhanced chemiluminescence signals with the use of ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Additional antibody dilutions used included CaMKII2 and CaMKII2 antibody at 1:1,000, polyclonal antibody for phosphorylated CaMKII2 at 1:500, monoclonal HA and monoclonal Flag antibodies at 1:1,000, and phosphorylated PT17 at 1:5,000.

CaMKII activity assay. Thirty-six to forty-eight hours after infection with adenoviral constructs, 300 μl of lysis buffer (50 mM MOPS, pH 8.6, 100 mM Na\(_2\)PO\(_4\), 100 mM NaF, 250 mM NaCl, 2 mM Na\(_2\)VO\(_4\), 3 mM EGTA, and 1% NP-40) was added to each 60-mm dish. Lysates were scraped and centrifuged at 14,000 rpm for 10 min at 4°C. Total CaMKII activity in the presence of saturating Ca\(^{2+}\) and calmodulin, as well as autonomous activity, measured in the absence of activators, was determined as described previously using autocamtide II, a specific peptide substrate (1).

Cell migration assays. Cultured VSM cells were grown in medium containing 10% FBS (as described in Cell culture) for 36–48 h after infection with adenoviral constructs. All 60-mm dishes were washed once in HBSS, trypsinized, and seeded at 150,000 cells/can-modified Boyden chamber (PET track-etched, 8-m pore, 12-well format; Becton Dickinson, San Diego, CA) coated with 3 μg/ml fibronectin (Sigma). Chambers were prepared by adding to the lower chambers medium containing either DMEM with 0.4% FBS (control) or 10 ng/ml PDGF-BB in DMEM with 0.4% FBS. Cells were permitted to migrate for 4 h. The tops of the membranes were swabbed to remove nonadherent cells on the bottom surfaces, membranes were fixed for 30 min in PBS containing 4% parafomaldehyde (pH 7.4). Cells were stained for 10 min in Coomassie blue. Filters were removed from the chamber and mounted onto microscope slides on which four
identically located fields per membrane were averaged for quantification of cell number.

Adhesion assay. Thirty-six to forty-eight hours after infection with adenoviral constructs, the ability of the cells to adhere to a fibronectin-coated surface (10 μg/ml; Sigma) was assayed as previously described (3).

Statistical evaluations and comparisons. All data are expressed as means ± SE. Mean values of groups were compared by ANOVA with post hoc comparisons using the Newman-Keuls test. For all comparisons, P < 0.05 was considered statistically significant.

RESULTS

Expression and characterization of CaMKIIδ2 mutants. CaMKIIδ2 is the major subunit expressed in cultured rat aortic VSM cells (20). To probe the function of this subunit, two HA-tagged mutants were produced and expressed in VSM cells by using replication-deficient adenoviruses (Fig. 1A). One virus (Ad-KN:δ2) contained a kinase-negative construct as a result of amino acid substitution (K43A) that prevents the binding of ATP (17). The second adenovirus (Ad-CA:δ2) contained a constitutively active construct produced by a charged amino acid substitution at T287D, which mimics autophosphorylation at that site and results in autonomous activity in the absence of bound Ca2+/calmodulin (24).

Preliminary experiments using adenoviruses to introduce green fluorescent protein (GFP) into VSM cells established that infection efficiencies in these cells were essentially 100% (data not shown). Increasing the MOI of both Ad-KN:δ2 and Ad-CA:δ2 resulted in expression of mutant δ2-subunits that resolved slightly larger than the endogenous isoform because of addition of the NH2-terminal HA tag. Infection with adenovirus itself did not significantly alter endogenous levels of CaMKIIδ2, as demonstrated by addition of increasing MOI of Ad-βGal (Fig. 1B).

To determine the effects of expressing these constructs on CaMKII activity, in vitro kinase assays (1) were performed using lysates of control and infected cells (Fig. 2). CaMKII activity was assayed both with and without added Ca2+ and calmodulin to obtain total and autonomous kinase activity, respectively. Infection of cells with 10 MOI of Ad-KN:δ2, which produced approximately fivefold more mutant subunits relative to endogenous subunits (Fig. 1B), decreased total CaMKII specific activity in VSM cell lysates by ~50%. Basal autonomous activity was not detected in cells infected with 10 MOI of Ad-KN:δ2 (Fig. 2). Conversely, comparable increases in the expression levels of the constitutively active δ2 mutant after infection with 10 MOI of Ad-CA:δ2 significantly increased both autonomous and total CaMKII activity in VSM cell lysates (Fig. 2). The level of autonomous activity observed with this MOI of active δ2 is comparable to the total Ca2+/calmodulin-stimulated activity in control cells. Infection with Ad-βGal did not significantly alter CaMKII specific activity.

To test the actions of mutant constructs on CaMKII activity within intact VSM cells, phosphorylation of a known substrate for CaMKII was evaluated after stimulation of cells with a Ca2+-dependent stimulus (ionomycin). Because endogenous substrates for CaMKII in VSM cells have not yet been well defined, we overexpressed PLB, which is known to be phosphorylated by CaMKII specifically on Thr17 (12, 22) and has been used as a readout of CaMKII activity in the heart (11, 36). PLB is not expressed significantly in these cultured VSM cells (data not shown); therefore, a Flag-tagged PLB cDNA was engineered into an adenovirus (Ad-PLB) for high-efficiency infection and expression. Infection with Ad-PLB resulted in PLB protein expression in the cells, as detected by immunoblotting with an anti-Flag antibody (Fig. 3, A and B). In VSM cells coinfected with control Ad-βGal (MOI = 10) and Ad-PLB (MOI = 160), ionomycin stimulated a significant increase in the phosphorylation of PLB on Thr17 (Fig. 3B), which was inhibited by KN-93 pretreatment (Fig. 3A), validating this as a readout of endogenous CaMKII activity. Introduction of the kinase-negative δ2 mutant resulted in inhibition of ionomycin-stimulated Thr17 phosphorylation by 65 ± 10% (n = 3), indicating a dominant-negative function of the construct with respect to endogenous CaMKIIδ2-dependent substrate phosphorylation.

Introduction of Ad-CA:δ2 stimulated PLB Thr17 phosphorylation even in the absence of the ionomycin stimulus, consistent with the constitutive activity of the expressed kinase (Fig. 3B). Addition of a Ca2+ stimulus resulted in further phosphorylation of PLB, consistent with in vitro assays (Fig. 2) indicating that the T287D mutation confers only partial activation of the kinase in the absence of added Ca2+/calmodulin.

**Effects of CaMKIIδ2 mutants on Thr287 autophosphorylation.** Autophosphorylation of CaMKII on Thr287 has been shown to be important not only for calmodulin trapping and autonomous activity of the enzyme (9, 23) but also for targeting the holoenzyme (2). To determine the effects of overexpressing the CaMKIIδ2 mutants on Thr287 autophosphorylation, VSM cells were stimulated with ionomycin and the lysates were immunoblotted with an antibody that specifically recognizes phosphorylated Thr287, as previously described (31). In uninfected cells (Fig. 4, A and B) or in cells infected with...
with Ad-βGal (MOI = 10) (Fig. 4B), stimulation with ionomycin resulted in transient Thr 287 autophosphorylation, which peaked 45 s–1 min after the addition of ionomycin and fell to near basal levels after 5 min. Pretreatment of VSM cells for 30 min with the pharmacological inhibitor of CaMKII, KN-93, attenuated this autophosphorylation event (Fig. 4A). Conversely, infection with Ad-KN:δ2 (MOI = 10) did not inhibit ionomycin-stimulated Thr 287 autophosphorylation. In fact, expressed kinase-negative subunits became phosphorylated on Thr287 in parallel with the endogenous subunits (Fig. 4B). The overexpression of constitutively active CaMKII δ2 after infection

Fig. 2. CaMKII δ2 mutant overexpression affects kinase activity in vitro. CaMKII activity in vascular smooth muscle (VSM) cell lysates after infection with Ad-KN:δ2 or Ad-CA:δ2 was assayed in vitro using a selective peptide substrate, autocamtide II (1). After infection with 10 multiplicities of infection (MOI) of Ad-KN:δ2, total CaMKII (+Ca 2+/CaM) activity was reduced to 50% of that in control lysates. Infection with 10 MOI of Ad-CA:δ2 resulted in a significant increase in autonomous CaMKII activity assayed in the absence of Ca 2+/CaM. Infection of cells with control Ad-βGal (≤100 MOI) had no significant effect on CaMKII activity. Results are means of 2 determinations in 2 separate experiments.

Fig. 3. CaMKII δ2-dependent phosphorylation of phospholamban (PLB) in cultured VSM cells. Cultured VSM cells were infected with an adenovirus containing a Flag-tagged PLB at 320 (A) or 160 MOI (B). Expression was detected by immunoblotting with anti-Flag antibody (IB: Flag). CaMKII-dependent phosphorylation of PLB was detected by immunoblotting with an antibody recognizing the phosphorylated Thr17 residue (IB: PT17 PLB) of immunoprecipitated PLB. A: cells expressing the Ad-PLB construct were stimulated with ionomycin for 45 s, and phosphorylation on T17 of PLB was detected. The level of phosphorylation was attenuated by pretreating the cells for 30 min with 30 μM KN-93, the pharmacological inhibitor of CaMKII. B: cultured VSM cells were coinfected with 160 MOI of Ad-PLB and 10 MOI of Ad-KN:δ2, Ad-CA:δ2, or control Ad-βGal. Expression of mutant CaMKII δ2 constructs was detected by immunoblotting with anti-hemagglutinin (HA) antibody. Expression of kinase-negative CaMKII δ2 (K43A) attenuated ionomycin-stimulated phosphorylation of PLB. Expression of the constitutively active CaMKII δ2 (T287D) caused a significant increase in PLB Thr17 phosphorylation in the absence of a Ca 2+ -mobilizing stimulus. Blots are representatives of 3 separate experiments.

Fig. 4. CaMKII δ2 mutant overexpression affects autophosphorylation of CaMKII δ2 subunits. Immunoblot analysis with an antibody that specifically recognizes the autophosphorylated Thr287 residue of CaMKII (IB: phospho-CaMKII) after treatment of cells with the Ca 2+ ionophore ionomycin is shown. A: cultured VSM cells were treated with ionomycin for 45 s, resulting in an increase in Thr287 phosphorylation. Pretreatment with the pharmacological inhibitor KN-93 (30 μM) for 30 min before stimulation caused attenuation of autophosphorylation. B: in cells infected with Ad-KN:δ2 (MOI = 10), ionomycin stimulated autophosphorylation of overexpressed kinase-negative subunits. In cultured cells infected with Ad-CA:δ2 (MOI = 10) and expressing constitutively active CaMKII δ2, ionomycin-stimulated autophosphorylation of endogenous δ2-subunits was blocked. Infection with control adenovirus (Ad-βGal; MOI = 10) demonstrated no effects on CaMKII δ2 subunit autophosphorylation in response to ionomycin stimulation. All blots shown are representatives of at least 4 separate experiments.
tion with Ad-CA:δ2 (MOI = 10) blocked ionomycin-stimulated autophosphorylation of Thr287 in the endogenous subunits and, because of the T287D mutation, was not itself a substrate for intersubunit autophosphorylation reactions (Fig. 4B).

Kinase-negative or constitutively active CaMKIIδ2 has no effect on VSM cell adhesion. To determine whether VSM cell adhesion was affected by the mutant δ2 constructs, cells were infected with either Ad-KN:δ2 or Ad-CA:δ2, and their adherence to fibronectin-coated dishes was assayed (3). As shown in Figure 5, VSM cell adherence increased for 2 h, and overexpression of either the kinase-negative or constitutively active mutant had no significant effects on adherence, suggesting that the effects of each mutant on cell migration in the modified Boyden chamber assay (described below) were not due to changes in adhesion efficiency.

Effects of kinase-negative and constitutively active CaMKIIδ on VSM cell migration. The effects of overexpressing mutant CaMKIIδ2 constructs on VSM cell migration were evaluated in a modified Boyden chamber assay by using PDGF-BB (Fig. 6A) as a chemotactic stimulus. In control VSM cells infected with 10 MOI of Ad-βGal, addition of PDGF to the lower chamber stimulated a threefold increase in the number of cells migrating to the lower side of the membrane. Expression of the kinase-negative CaMKIIδ2 subunit, which inhibited endogenous CaMKII activity by 65% (Fig. 3), significantly increased the number of cells migrating to the lower chamber under unstimulated conditions and potentiated the response to PDGF (P < 0.05; Fig. 6, A and B). In contrast, pretreatment with KN-93, a purportedly selective CaMKII inhibitor, at concentrations previously shown to maximally inhibit CaMKIIδ2 activity in these cells (1, 31), significantly inhibited PDGF-induced VSM migration in control cells (Fig. 7). Similar results were found with the use of a mixed stimulus (conditioned medium) for chemotaxis (data not shown). Furthermore, acute overexpression of the constitutively active CaMKIIδ2 mutant significantly decreased the number of cells migrating in response to PDGF (P < 0.05; Fig. 6, A and B). The inhibitory

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**Fig. 6.** CaMKIIδ2 mutant overexpression affects chemotactic VSM cell migration. VSM cells were infected with 10 MOI of Ad-βGal, Ad-KN:δ2, or Ad-CA:δ2. After 36 h, cell migration was assayed using a modified Boyden chamber approach. A: representative fields of infected VSM cells after 4 h migration in response to no stimulus (0.4% FBS) or to 10 ng/ml PDGF-BB added to the lower chamber. Cells on the lower surface of the membrane were stained with Coomassie blue. B: quantification of VSM cell migration in unstimulated controls and after addition of 10 ng/ml PDGF-BB to the lower chamber for 4 h. Values are means ± SE for 3 separate experiments with Ad-KN:δ2- and Ad-CA:δ2-infected cells (n = 6) and for 6 separate experiments with Ad-βGal control (n = 12). *P < 0.05, PDGF treated compared with unstimulated; †P < 0.05 compared with Ad-βGal controls.

**Fig. 5.** Adhesion of VSM cells expressing CaMKIIδ2 mutants. Cultured VSM cells were infected with 10 MOI of Ad-βGal, Ad-KN:δ2, or Ad-CA:δ2. After 36 h, the cells were suspended, and adherence to fibronectin-coated dishes was assayed for 2 h. Adherent cells were stained with toluidine blue and lysed, and absorbance at 595 nm was determined. The mutant CaMKIIδ2 constructs did not affect cell adhesion to fibronectin significantly at the assayed time points (n = 6 determinations). Values are means ± SE for 3 separate experiments.

**DISCUSSION**

Although all CaMKII isoforms have conserved catalytic and regulatory domains and therefore have similar kinetic properties, recent studies have indicated significant isoform-dependent differences in subcellular localization and protein targeting (2, 21, 30). Therefore, in attempting to understand CaMKII function in a given cell type, it is important to consider the isoform expression pattern. We (20) previously identified the
δ2-isofrom of CaMKII as the major subtype within cultured rat aortic VSM cells. To determine specific functions of this subunit, in this study we validated and applied molecular approaches by using CaMKIIδ2 mutants with altered regulatory properties to manipulate cellular CaMKII activity. Because this study, to our knowledge, is the first functional assessment of a kinase-negative CaMKII construct in any cell, the effects of expressing this mutant on endogenous CaMKII activity, including autophosphorylation reactions, were carefully characterized. To provide an unambiguous readout of endogenous or overexpressed kinase activity, PLB Thr17 phosphorylation was used as a specific substrate (11, 36). Our results indicate phosphorylation of PLB by endogenous CaMKII and inhibition of this activity with KN-93 or approximately fivefold overexpression of the kinase-negative CaMKIIδ2 mutant relative to endogenous kinase. Furthermore, this level of kinase-negative mutant expression inhibited CaMKII activity in VSM lysates assayed in vitro. Thus the results of both approaches indicate that overexpression of this construct is dominant negative with respect to CaMKII substrate phosphorylation.

Analysis of CaMKIIδ2 phosphorylation on Thr287, however, indicated that overexpression of the kinase-negative mutant at this level did not block the highly cooperative (17, 23) autophosphorylation reactions and, in fact, that it served as a substrate for these reactions (Fig. 4). Because the kinase-negative subunit is unable to catalyze autophosphorylation, and because these reactions have been demonstrated to be inter-subunit within the multimeric holoenzyme (4), these data indicate that the mutant subunit integrates into holoenzymes with endogenous δ2-subunits and suggest proper targeting of the mutant subunits. Conversely, in cells overexpressing the constitutively active mutant, endogenous CaMKIIδ2 Thr287 autophosphorylation was inhibited. This result has a number of potential explanations, including inactivating autophosphorylations catalyzed by the mutant on Thr287 in the calmodulin binding domain (19) and/or upregulated phosphatase activities enhancing reversal of the autophosphorylation events. Regardless of the mechanisms involved, overexpressing these mutants in Thr287 autophosphorylation could be an important consideration in interpreting the functional effects of these mutants, as in the present study and in previously published experiments in which acute (13) or stable (15, 35) overexpression approaches were used.

Several previous reports have indicated a role for CaMKII in VSM cell migration (13, 15, 35). A consistent finding confirmed in the present study is that selective pharmacological inhibitors of CaMKII (KN-62 or KN-93) inhibit migration of VSM cells toward chemotactic stimuli (13, 15). These agents act by interfering with Ca2+/calmodulin binding and subsequent activation, including autophosphorylation reactions (Fig. 4A). In the present study, however, introduction of the kinase-negative CaMKIIδ2 mutant into VSM cells enhanced migration toward PDGF (Fig. 6) or conditioned medium. We currently have no definitive explanation for this discrepancy in results with the use of the different approaches. Potential explanations are that KN-93 exerts nonspecific effects on VSM cell migration or that the kinase-negative construct enhances migration through an effect unrelated to CaMKII per se. Another, more interesting explanation that is consistent with both the pharmacological and molecular approaches is that the requirement for CaMKII activation in cell migration necessitates autophosphorylation.

A requirement for CaMKII autophosphorylation in cell migration is also consistent with the results obtained with overexpression of the constitutively active CaMKIIδ2 mutant. In these experiments, infection with constitutively active T287D mutant was titrated to achieve a level of expression that resulted in autonomous activity that was quantitatively comparable to the maximum levels of endogenous Ca2+/calmodulin-stimulated activity expressed in normal cells (Fig. 2). As discussed above, however, the effect of overexpressing this mutant on autophosphorylation of endogenous subunits was inhibitory, and the net effect on VSM cell migration also was inhibitory.

Similar studies using overexpressed constitutively active CaMKII mutants have been published, but with different conclusions. In general, the prior studies found enhanced VSM cell migration (35) or rescue of KN-62 or KN-93 inhibition of PDGF-stimulated cell migration (13, 15). Differences between the current and prior studies may factor into why essentially opposite results were found in our study. In many of the previous studies, the constitutively active construct was stably expressed (15, 35). It could be expected that this approach would result in significant adaptive changes, including the induction of phosphatase activity, as indicated by experiments evaluating CaMKII in VSM cell migration in response to wounding (35). With the use of the more acute adenoviral infection approach, as in the present study, there is less time for such adaptive changes to occur. In addition, in those prior experiments in which constitutively active CaMKII constructs were overexpressed using an adenoviral delivery system, the constructs were mutants of the CaMKIIα isoform (13) and contained a second charged residue substitution (V287D) in addition to the charge substitution at the autophosphorylation site in the α-subunit (T287D). To the extent that unique sequences in the δ2-isoform and/or autophosphorylation levels may be important in holoenzyme targeting, the more densely
charged, constitutively active α-subunit may act differently from the constitutively active δ2-construct used in the present study. A potential requirement for CaMKII autophosphorylation in VSM cell migration (discussed above) might be satisfied by the more densely charged mutant and not by the δ2-mutant, which also inhibited endogenous subunit autophosphorylation. Finally, an important limitation of any experiment expressing a constitutively active multifunctional serine/threonine kinase is persistent, unregulated phosphorylation of normal substrates as well as recruitment of nonphysiological substrates.

With the use of new molecular approaches, the present study indicates an important role for CaMKIIδ2 in regulating cultured VSM cell migration. If interpreted strictly in the context of CaMKIIδ2 activity toward substrates other than itself, the results are not consistent with the effects of chemical inhibitors of the kinase. Alternatively, an inclusive interpretation of the results could point to a requirement for CaMKII autophosphorylation in VSM cell chemotactic migration. Although it is speculation at this point, this possibility is consistent with an increasing number of results in other systems indicating the importance of autophosphorylation events in directing CaMKII holoenzyme targeting (2) and enzymatic activity (34). The ultimate cellular mechanisms underlying CaMKII actions on cell migration are also not yet known but could be related to effects of the kinase on the stability and turnover of focal adhesions (32) or on the activity of the contractile apparatus, perhaps by inhibiting myosin light chain kinase activity (28). The present results indicate the need for additional studies to clarify the mechanisms by which CaMKIIδ2 regulates VSM cell migration and whether these results can be extrapolated to other cell systems.

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