Regulation of a Calcium-dependent Tyrosine Kinase in Vascular Smooth Muscle Cells by Angiotensin II and Platelet-derived Growth Factor

DEPENDENCE ON CALCIUM AND THE ACTIN CYTOSKELETON

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A novel, p125FAK homologue, CADTK, has been detected in neural, epithelial, or hematopoietic cells but not in fibroblasts. We now demonstrate CADTK expression in a mesenchymal cell, rat aortic smooth muscle cells (RSMC). Angiotensin II (Ang II) or platelet-derived growth factor (PDGF-BB and PDGF-AA) markedly stimulated CADTK tyrosine phosphorylation in RSMC but did not affect p125FAK phosphorylation. The PDGF-dependent CADTK tyrosine phosphorylation was slower and more prolonged than that of Ang II, correlating well with the differential effects of these agonists on cytosolic calcium (Ca^{2+}) signaling. An intracellular calcium chelator inhibited both the rapid and sustained activation of CADTK by Ang II and PDGF. Extracellular calcium chelation inhibited the PDGF-stimulated increase in CADTK tyrosine phosphorylation as well as the sustained (but not the early) activation by Ang II. In contrast, p125FAK tyrosine phosphorylation was maximal in quiescent, adherent RSMC and was not affected by incubation with EGTA. Depletion of protein kinase C activity partially inhibited both the Ang II- and PDGF-induced CADTK tyrosine phosphorylation. Additional results confirm a relation between CADTK and the cytoskeleton. First, the tyrosine phosphorylation of paxillin correlated with activation of CADTK; this increase was inhibited by EGTA. Second, cytochalasin D blocked the PDGF- or Ang II-stimulated tyrosine phosphorylation of CADTK, suggesting a role for the cytoskeleton in agonist-dependent CADTK activation. Third, immunofluorescence analysis of CADTK localization demonstrated actin-like cytoskeleton staining extending into focal contacts. These results suggest that in mesenchymal cells, CADTK is localized to and activated by an actin cytoskeleton-dependent mechanism; a mechanism that is regulated in a calcium and protein kinase C-dependent manner independently of p125FAK.

The migration and proliferation of vascular smooth muscle cells are key events in the development of atherosclerotic lesions and restenosis following angioplasty (reviewed in Ref. 1). In addition to the pivotal role these cells play in the etiology of atherosclerosis, vascular smooth muscle cells also contribute to the pathology of hypertension (2). Smooth muscle cells respond to growth factors such as the polypeptide PDGFs (3) (PDGF-BB or PDGF-AA), which initiate intracellular signaling pathways leading to either cell proliferation or migration (reviewed in Ref. 3). Binding of PDGF to its cognate receptor tyrosine kinase stimulates a plethora of events including activation of PI-3 kinase, Ras/Raf signaling, and phospholipase Cγ (reviewed in Ref. 4). Increasing phospholipase Cγ activity further contributes to intracellular signaling by regulating cytosolic calcium levels and protein kinase C (PKC) activity. However, despite considerable knowledge of these initial events, the ultimate effect of these signals on smooth muscle function remains to be elucidated.

Angiotensin II (Ang II) is a component of the renin-angiotensin signaling pathway and is important for both the normal and pathological (i.e. hypertensive) states of smooth muscle physiology (reviewed in Ref. 5). Ang II is a growth factor for smooth muscle and other cell types (6). In rat smooth muscle cells (RSMC), Ang II signaling is mediated by the AT1 receptor, a seven-transmembrane receptor coupled to the G_{s} subtype of heterotrimeric G proteins (7). Activation of this receptor increases phospholipase Cβ activity, resulting in the increase of cytosolic free calcium and protein kinase C activity (6). Ultimately, the influence of PDGF or Ang II on intracellular calcium homeostasis affects the regulation of vascular tone, migration, and the growth of smooth muscle cells (reviewed in Ref. 8).

Ang II has been shown to increase protein tyrosine phosphorylation in smooth muscle and other cell types. This has been variably attributed to the activation of p125FAK (9), Src (10), a calcium/calmodulin-dependent tyrosine kinase (11), or other unidentified tyrosine kinases (12). Recently, a calmodulin-independent, calcium-dependent tyrosine kinase (CADTK) was purified from Ang II-activated rat liver epithelial cells (13). Peptide sequencing, isolation of the cDNA, and deduction of the primary amino acid sequence demonstrated that CADTK was identical to the kinase recently identified by others and known

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1 The abbreviations used are: PDGF, platelet-derived growth factor; RSMC, rat aortic smooth muscle cell(s); MAP, mitogen-activated protein; BAPTA-AM, bis(o-aminophenoxy)ethane-N,N,N′,N″-tetraacetic acid tetraacetoxymethyl ester; CADTK, calcium-dependent tyrosine kinase; TPA, 12-tetradecanoylethorhol 13-acetate; Ang, angiotensin; TBS, Tris-buffered saline/Tween; PKC, protein kinase C; p125FAK, p125 focal adhesion kinase; pp60 src, p90 ribosomal S6 kinase, HBSS, Hank’s buffered saline solution; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence.
Regulation of CADTK in Vascular Smooth Muscle Cells

as Pyk-2 (14), CAKβ (15), RAFTK (16), or FAK2 (17).

CADTK contains a high degree of sequence homology to the focal adhesion kinase (p125<sub>FAK</sub>), including conservation of a number of potentially important tyrosine phosphorylation sites (14). One of these, tyrosine 402, is analogous to tyrosine 397 of p125<sub>FAK</sub> and is the primary site of autophosphorylation in this kinase (18). Phosphorylation of this amino acid provides a binding site for kinases containing SH2 domains such as Src and Fyn (18), and phosphorylation of Tyr<sup>402</sup> in RAFTK (CADTK) results in binding of the Src family kinases Lck and Fyn to this residue (19). Pyk-2/CADTK may be responsible for linking Src kinase signaling to “downstream” signaling events such as the activation of MAP kinase (20), although a link between CADTK and MAP kinase activation is not abs in rat epithelial cells (13). Other studies have suggested that CADTK may be “upstream” of the c-Jun NH<sub>2</sub>-terminal kinase (Jnk) (13, 21) and p70 S6 kinase (22); however, the requirement for Src in the activation of these kinases has not been established.

The expression of CADTK/Pyk-2/CaKβ/RAFTK has been demonstrated in a number of cell types including those of neural (14), hematopoietic (16), or epithelial origin (13). In contrast, this kinase is not expressed in fibroblasts (15). Specifically, CADTK has been shown to be regulated in a calcium- and PKC-dependent manner in liver epithelial (13), PC12 (14), megakaryocytes (23), platelets (24), and B cells (25) by G protein-coupled agonists and other extracellular stimuli. Potential targets for phosphorylation by this enzyme include the cytoskeletal proteins paxillin (23, 26, 27) and p130Cas (27), and protein-coupled agonists and other extracellular stimuli. Potential targets for phosphorylation by this enzyme include the cytoskeletal proteins paxillin (23, 26, 27) and p130Cas (27), and protein-coupled agonists and other extracellular stimuli. Potential targets for phosphorylation by this enzyme include the cytoskeletal proteins paxillin (23, 26, 27) and p130Cas (27), and protein-coupled agonists and other extracellular stimuli. Potential targets for phosphorylation by this enzyme include the cytoskeletal proteins paxillin (23, 26, 27) and p130Cas (27), and protein-coupled agonists and other extracellular stimuli. Potential targets for phosphorylation by this enzyme include the cytoskeletal proteins paxillin (23, 26, 27) and p130Cas (27), and protein-coupled agonists and other extracellular stimuli.

RESULTS

Expression and Regulation of CADTK by Ang II or PDGF in RSMC—CADTK expression was examined in RSMC by Northern and Western blot analysis. Total RNA was isolated from RSMC and subjected to Northern blot analysis using a 4.0-kilobase transcript was found that was identical in size to the CADTK mRNA expressed in rat liver epithelial cell lines (GN4, WB). To confirm that CADTK protein was expressed in RSMC, cell lysates were immunoprecipitated with antisera specific for CADTK and immunoblotted for CADTK protein. Immunoblotting RSMC lysates for CADTK detected a 120-kDa protein that co-migrated on SDS-PAGE with CADTK cDNA labeled with [3H]dCTP by random priming.

EXPERIMENTAL PROCEDURES

Materials—PDGF-AA and PDGF-BB were purchased from Life Technologies, Inc. Ang II was obtained from Sigma and diluted in 50 mM acetic acid. RC20H anti-Tyr(P) antibody was purchased from Transduction Laboratories and diluted 1:5000 in TBS (including 1% bovine serum albumin) for immunoblotting experiments. Paxillin monoclonal antibody was purchased from Transduction Laboratories. Protein A beads were purchased from Bio-Rad and washed three times with phosphate-buffered saline prior to use. 12-Tetradecanoylphorbol 13-acetate (TPA) and BAPTA-AM, were obtained from BioLoy and dissolved in Me<sub>2</sub>SO prior to the addition to cells. RSMC were obtained from the laboratory of Dr. Russel Ross. These cells were isolated from thoracic aorta of male Sprague-Dawley rats (4 months old) and prepared as described previously (28).

Cell Cultures—Subconfluent RSMC were maintained in Dulbecco's modified Eagle's medium with 10% bovine calf serum and penicillin/streptomycin in a humidified, 5% CO<sub>2</sub> atmosphere. 48 h prior to experiments, the medium was removed and replaced with medium lacking bovine calf serum. Following drug treatment, cells were washed twice with cold phosphate-buffered saline and harvested in 1 ml of immunoprecipitation buffer (20 mM Tris, pH 7.5, 1% Triton, 10% glycerol, 157 mM NaCl, 2 mM EDTA, 50 μM leupeptin, 10 μM calyculin A, 250 μM phenylmethylsulfonyl fluoride, and 150 μM sodium vanadate). The cell lysates were centrifuged at 12,000 × g for 10 min, and the resultant supernatants were transferred to clean microcentrifuge tubes.

Measurement of CADTK, p125<sub>FAK</sub>, and Paxillin Tyrosine Phosphorylation—The amount of CADTK, p125<sub>FAK</sub>, or paxillin tyrosine phosphorylation was assayed by the procedure described earlier (13, 29) with minor modifications. CADTK was immunoprecipitated with rabbit antisera developed against a GST-CADTK fusion protein (13). p125<sub>FAK</sub> was immunoprecipitated with a mouse monoclonal antibody, 2A7, kindly provided by Dr. T. Parsons (University of Virginia) or a polyclonal antibody, A17 (Santa Cruz). A monoclonal antibody (Transduction Laboratories) was used to immunoprecipitate paxillin. Protein A-agarose beads were used to precipitate the CADTK and A17 immunocomplexes, while protein A/G beads were used to precipitate the 2A7 and paxillin immunocomplexes. For determination of tyrosine phosphorylation, samples were applied to SDS-PAGE (10% acrylamide), transferred to polyvinylidene difluoride (Immobilon-P) membrane, blocked with 5% bovine serum albumin/TBST, immunoblotted with RC20H, and developed with enhanced chemiluminescence (ECL) reagent according to the manufacturer's protocol (NEN Life Science Products).

Northern Analysis—Total RNA was prepared from RSMC and rat liver epithelial cells (WB), with Trizol solution according to the manufacturer's protocol (Life Technologies, Inc.). RNA (30 μg) was separated on a 12% formaldehyde-agarose gel and transferred to a Zeta Probe® GT blotting membrane (Bio-Rad) and probed with full-length CADTK cDNA labeled with [32P]dCTP by random priming.

Determination of Intracellular Calcium—Ca<sup>2+</sup> was measured using digitized video microscopy in RSMC grown on glass coverslips and loaded with the calcium-sensitive fluorophore, Fura-2/AM, as described previously (30).

Immunofluorescence Detection of CADTK—For immunofluorescence microscopy, cells were grown on two-well chamber slides (Becton Dickinson) and fixed once with phosphate-buffered saline and 4% paraformaldehyde for 8 min. The cells were then permeabilized by washing with −20 °C acetone twice for 30 s. Cells were then washed three times with HBSS and blocked for nonspecific sites with 10% goat serum in HBSS for 1 h. Upon completion of blocking, cells were washed twice with HBSS and stained for filamentous actin using 1 μg/ml tetramethylrhodamine isothiocyanate (TRITC) phallolidin (Sigma) or an appropriate dilution of primary antibody in HBSS plus 10% goat serum and incubated for 1 h at room temperature. The antisera used were raised against a GST-CADTK fusion protein encompassing amino acids 1–80 and 680–860 of rat CADTK. The antiserum was prepared for immunofluorescence by purification of the IgG fraction using a Protein A-Sepharose column (Sigma) and was subsequently depleted of GST immunoreactivity by passing over an AFS-Gel 10 column made by coupling GST (18 mg) to this resin. The specific immunofluorescence was blocked by preincubation of the purified antibodies with the immunizing GST-CADTK fusion proteins. Cells were washed extensively and incubated in with a 1:500 dilution of secondary antibody labeled with Oregon Green (Molecular Probes). Fluorescence was visualized using a Zeiss Axioskop microscope under ×60 and ×100 objectives (×10 oculars) and photographed with Tmax 400 film.
Regulation of CADTK in Vascular Smooth Muscle Cells

The regulation of CADTK tyrosine phosphorylation (Tyr(P)) by agonists known to influence smooth muscle growth or migration was examined. CADTK Tyr(P) has been shown to increase in parallel with CADTK kinase activity (Poly-Glu/Tyr phosphorylation) (13); thus, CADTK Tyr(P) was determined as a measure of CADTK activity. However, it should be noted that the observed CADTK Tyr(P) measured in these studies may be a combination of that catalyzed by CADTK (autophosphorylation) and that of a CADTK-associated kinase such as Src. Both Src and Src family members (Lck, Fyn) have been shown to bind CADTK (Pyk-2/RAFTK (18, 19)) and may contribute to the phosphorylation of CADTK in cells.

Immunoprecipitation of CADTK from Ang II (1 μm)-treated cells demonstrated a rapid increase in CADTK Tyr(P) as determined by anti-phosphotyrosine (anti-Tyr(P)) immunoblotting (Fig. 1B). An increase in CADTK phosphorylation was detected as early as 30 s following the Ang II addition. This increase peaked after approximately 3–5 min and began to decline thereafter. PDGF-BB also stimulated a significant increase in CADTK phosphorylation, although the peak of phosphorylation in response to this stimulus occurred later than that observed with Ang II. Again, CADTK Tyr(P) was observed as early as 30 s after exposure to PDGF-BB; however, the tyrosine phosphorylation of CADTK continued to increase up to 20 min after the PDGF-BB addition and declined very slowly thereafter (Fig. 1, B and C, and data not shown). The increase in CADTK Tyr(P) was also accompanied by an increase in tyrosine kinase activity as assessed by phosphorylation of poly-Glu/Tyr in the presence of [γ-32P]ATP/MgCl2 (data not shown). PDGF-AA also stimulated an increase in CADTK Tyr(P), but consistent with a higher number of receptors for PDGF-BB (PDGF-β) as compared with PDGF-AA (PDGF-α) in these cells, PDGF-BB gave a larger increase in CADTK phosphorylation than that observed with an identical concentration (1.5 nM) of PDGF-AA (data not shown).

**p125FAK Tyrosine Phosphorylation Is Not Stimulated by Ang II or PDGF in RSMC**—The primary amino acid sequence of CADTK contains regions of high homology to p125FAK (65% amino acid similarity overall) (31). Because of the high degree of similarity between these kinases, we compared the effect of stimuli found to increase CADTK phosphorylation on the tyrosine phosphorylation of p125FAK in RSMC. Immunoprecipitation and anti-Tyr(P) blotting of p125FAK from lysates of adherent, serum-starved cells showed that p125FAK was highly tyrosine-phosphorylated in quiescent RSMC in contrast to CADTK, which contained little or no detectable phosphotyrosine under these conditions (Fig. 1A, compare B and D). Incubating RSMC with Ang II or PDGF-BB did not stimulate a significant increase in p125FAK Tyr(P), even after prolonged exposure to Ang II (10 min) or PDGF (20 min) (Fig. 1D and data not shown). In contrast, these agonists markedly stimulated CADTK Tyr(P) (see Fig. 1B), demonstrating that the phosphorylation of CADTK was regulated by these agonists independently of p125FAK in RSMC.

**Calcium Chelators Inhibit CADTK but Not p125FAK Phosphorylation**—BAPTA-AM is a cell-permeable calcium chelator that prevents the hormonally induced rise in intracellular calcium and inhibits the agonist-stimulated CADTK/Pyk-2 activity in PC12 or rat liver epithelial cells (14). CADTK is not tyrosine phosphorylation (α-P-TYR) or p125FAK protein (α-FAK) was measured by SDS-PAGE/immunoblotting.
activated by calcium or calmodulin in vitro (29), suggesting that CADTK is regulated by a calcium-dependent event in intact cells. The calcium dependence of agonist-stimulated CADTK phosphorylation in RSMC was examined by incubating cells with BAPTA-AM (50 μM), or EGTA (2 mM), a non-cell-permeable calcium chelator. As shown in Fig. 2A, treatment with BAPTA-AM or EGTA inhibited both the PDGF-BB and Ang II-stimulated increase in CADTK Tyr(P). However, incubating RSMC with EGTA did not inhibit the initial, rapid increase in Ang II-stimulated CADTK (30–90 s) phosphorylation but significantly reduced the latter phase of tyrosine phosphorylation (10–20 min) (Fig. 2B), suggesting that at later times, influx of calcium from extracellular sources played a more important role in the activation of CADTK. In comparison, the cell-permeable chelator BAPTA-AM inhibited the early Ang II-dependent CADTK phosphorylation (see Fig. 2A), demonstrating the reliance of this initial event upon an intracellular calcium signal.

With PDGF-BB as a stimulus, EGTA inhibited both the initial and the latter phases of CADTK phosphorylation (Fig. 2C), indicating that both intracellular and extracellular sources of calcium were important for activation of CADTK in response to this agonist. The importance of extracellular calcium was further supported by incubation of RSMC in calcium-free media; this treatment inhibited the tyrosine phosphorylation of CADTK in response to PDGF or Ang II (10 min). Incubation of RSMC with the calcium ionophore ionomycin (1 μM, 10 min) increased CADTK phosphorylation, demonstrating the efficacy of a calcium signal alone to increase CADTK phosphorylation (data not shown).

In comparison, incubation with EGTA did not affect the tyrosine phosphorylation of p125FAK (Fig. 2D), demonstrating that the calcium-dependent regulation of CADTK occurred independently of p125FAK in RSMC.

**CADTK Regulation: Comparison of Chelators on Calcium Signaling in RSMC—PDGF and Ang II have been shown to have qualitatively different effects on the source, duration, and level of elevation of cytosolic free calcium in vascular smooth muscle cells (32).** To attempt to explain the differential effects of the calcium chelators on CADTK phosphorylation, calcium imaging was performed by incubating RSMC with a cell-permeable fluorescent indicator, Fura-2/AM, and the levels of PDGF- and Ang II-stimulated cytosolic free calcium ([Ca2+]i) were measured as described earlier (30). In serum-starved RSMC, Ang II stimulated a sharp increase in [Ca2+]i, which peaked after 15–30 s and declined to approximately 50% of the maximum level within 1–2 min (Fig. 3A). In contrast, PDGF increased [Ca2+]i, more slowly (maximum at 60–90 s), and the [Ca2+]i remained elevated for at least 10 min (Fig. 3B). As expected, pretreatment with BAPTA-AM abolished the initial PDGF or Ang II-stimulated peak of [Ca2+]i (data not shown). Incubation with the extracellular calcium chelator, EGTA, did not inhibit the initial Ang II-stimulated increase in [Ca2+]i, but eliminated the slower prolonged elevation of [Ca2+]i (Fig. 3A). With PDGF as a stimulus, EGTA partially inhibited the initial increase and completely eliminated the sustained increase in [Ca2+]i (Fig. 3B), indicating that the slower accumulation of [Ca2+]i was primarily due to influx from extracellular stores. These findings correlated well with the effect of these chelators on CADTK Tyr(P) (see Fig. 2, B and C), suggesting that the distinct pattern of CADTK activation by these stimuli may be through the regulation of both intracellular and extracellular calcium signaling.

**PKC-dependent Activation of CADTK Phosphorylation in RSMC—**Phorbol esters (TPA) stimulate PKC and CADTK/Pyk-2 activity in both rat epithelial and PC12 cells (13, 14). To investigate whether PKC was required for the PDGF- or Ang II-dependent phosphorylation of CADTK in RSMC, cells were chronically exposed to TPA (1 μM, 24 h) to deplete cells of TPA-sensitive isoforms of PKC. Acute incubation of non-PKC-depleted cells with TPA (5 min) resulted in an increase in CADTK Tyr(P) similar in magnitude to that found with Ang II or PDGF (Fig. 4). After chronic exposure to TPA (1 μM, 24 h), a brief treatment with TPA (100 nM, 5 min) did not increase CADTK Tyr(P), demonstrating the effectiveness of this treatment to ablate PKC activity. This protocol also eliminated the PKC-dependent activation of MAP kinase in these cells (data not shown). As shown in Fig. 4, down-regulation of PKC significantly inhibited both the Ang II- and PDGF-BB-stimulated increase in CADTK Tyr(P) (Fig. 4).

**Regulation of Paxillin Phosphorylation in RSMC—**The cy-
Regulation of CADTK in Vascular Smooth Muscle Cells

FIG. 3. Regulation of Ca^{2+} by Ang II or PDGF. Changes in cytosolic free calcium ([Ca^{2+}]) were measured in serum-starved RSMC immediately following exposure to Ang II (1 μM) (A) or PDGF-BB (1.5 nM) (B) as described under “Experimental Procedures.” [Ca^{2+}], was monitored using digitized videomicroscopy with Fura-2/AM as the fluorescent indicator. Ang II and PDGF were added at time 0, and EGTA (2 mM), when present, was added 10 min prior to agonist stimulation. The data presented are the average 340/380-nm ratio values obtained from single microscope fields containing an average of nine cells each. Results presented are representative of three or four independent experiments performed for each condition examined.

FIG. 4. CADTK Tyr(P) in PKC-depleted cells. Serum-starved RSMC were untreated (−TPA) or treated with TPA (+TPA, 1 μM, 24 h) prior to incubation with 100 nM TPA, 1 μM Ang II, or 1.5 nM PDGF-BB for 5 min. Cells were harvested in cell lysis buffer, and the amount of CADTK Tyr(P) was determined as described above.

Cytoskeletal protein paxillin has been shown to be phosphorylated in response to Ang II and PDGF in smooth muscle cells, presumably through the regulation of p125^{Fak} (9, 33, 34). Since our studies suggested that Ang II and PDGF activated CADTK, but not p125^{Fak}, we examined the ability of these agonists to affect paxillin tyrosine phosphorylation. Immunoblotting for anti-Tyr(P) immunoblotting demonstrated that treatment of RSMC with Ang II or PDGF-BB stimulated a significant increase in paxillin tyrosine phosphorylation over that found in quiescent cells (Fig. 5). The PDGF-stimulated increase in paxillin Tyr(P) was strongly inhibited by EGTA, whereas the Ang II-stimulated increase was inhibited to a lesser extent by this chelator; these results were consistent with a weaker effect of EGTA on Ang-dependent CADTK activation (Fig. 5). Similar results were also found with BAPTA-AM; however, in some experiments we observed partial inhibition of p125^{Fak} phosphorylation by this compound (data not shown). We therefore used EGTA, which inhibited CADTK, but not p125^{Fak} in these cells. As shown in Fig. 5, the ability of EGTA to prevent the PDGF-stimulated paxillin phosphorylation in response to PDGF was greater than that observed with Ang II and correlated with the stronger inhibitory effect of this compound on the PDGF-stimulated increase in CADTK activity. These experiments demonstrated that the changes in paxillin Tyr(P) correlated with the ability of compounds to increase (PDGF or Ang II) or decrease (EGTA) CADTK Tyr(P) but not p125^{Fak} Tyr(P) in these cells.

Cytochalasin D Treatment Prevents CADTK Activation—The carboxyl-terminal portion of CADTK contains high homology to the focal adhesion targeting domain of p125^{Fak} (14), suggesting that CADTK may be involved in focal adhesion and cytoskeletal signaling. This hypothesis has been further supported by recent studies demonstrating coimmunoprecipitation of CADTK with the cytoskeletal protein paxillin or with p130 Cas (26, 27). To investigate whether activation of CADTK required an intact cytoskeleton, cells were incubated briefly (10 min) with cytochalasin D (1 μM) prior to stimulation by various agonists. As shown in Fig. 6A, pretreatment with cytochalasin D prevented the increase in CADTK Tyr(P) stimulated by PDGF, Ang II, TPA, or ionomycin treatment (Fig. 6 and data not shown). Cytochalasin D did not inhibit CADTK Tyr(P) stimulated by the tyrosine phosphatase inhibitor pervanadate, nor was the recovery of CADTK protein compromised by cytochalasin D treatment (Fig. 6B).

CADTK Localizes to the Focal Adhesions and the Actin Cytoskeleton—To examine the relation of CADTK to cytoskeletal signaling, we examined the intracellular localization of CADTK by immunofluorescence. Serum-starved RSMC were plated on glass coverslips and fixed with paraformaldehyde, and CADTK was detected by incubation with the immunospecific antibodies as described under “Experimental Procedures.” Using two independent polyclonal antibodies raised against different regions of CADTK (amino acids 1–80 or 680–860 of rat CADTK), CADTK protein staining was found along actin microfilament-like structures that extended into focal adhesions (Fig. 7A). This filamentous-like staining co-localized in RSMC with rhodamine-conjugated phalloidin, indicating that these structures were actin filaments (Fig. 7, B and C). This is clearly a different staining pattern from p125^{Fak} and vinculin, which are densely concentrated around focal adhesion contacts in multiple cell types (35).
Regulation of CADTK in Vascular Smooth Muscle Cells

PDGF and Ang II are potent physiological mediators of vascular smooth muscle cell function, and many of the actions of these stimuli are likely to be mediated through increases in either calcium or PKC signaling. In this report, we demonstrate that the recently identified calcium- and PKC-activated tyrosine kinase, CADTK, is a likely candidate to mediate signal transduction by these stimuli in RSMC. This study confirms that both calcium and PKC are essential yet independent activators of CADTK phosphorylation. In addition, our results illustrate the importance of extracellular calcium in regulating CADTK in response to some agonists. The extended activation of CADTK by PDGF correlated well with the influence of this agonist on the influx of calcium from extracellular sources. Activation by PDGF was more sensitive to the extracellular calcium chelator EGTA than Ang II and supported earlier studies demonstrating the qualitatively different effects of these stimuli on calcium signaling (32).

The calcium chelators BAPTA-AM and EGTA did not completely inhibit the activation of CADTK by PDGF or Ang II, suggesting that an additional signal was required. Our studies suggest that this signal may be PKC. Down-regulation of PKC inhibited CADTK activation but did not compromise the ability of these agonists (e.g. PDGF, Ang II) to stimulate an increase in $[Ca^{2+}]_i$, indicating that the release of calcium from intracellular stores or influx from extracellular sources was not impaired by this treatment. Depletion of both PKC activity and calcium signaling (using calcium-free media) further reduced the activation of CADTK by Ang II, supporting the hypothesis that calcium and PKC are independent and potentially additive activators of CADTK. However, since calcium does not activate CADTK in vitro, the mechanism by which this and PKC increase CADTK activity in vivo remains to be elucidated; our results suggest that this is mediated through a cytoskeleton-dependent mechanism (see below).

We find that agonist-dependent phosphorylation of CADTK occurs independently of the highly homologous focal adhesion kinase, p125 FAK, in RSMC. These observations differ from previous studies in vascular smooth muscle cells that showed Ang II- and PDGF-stimulated p125 FAK tyrosine phosphorylation (9, 34, 36). In response to Ang II, Polte et al. (36) found approximately a 4-fold increase in p125 FAK Tyr(P), whereas a 50% increase in phosphorylation was observed by Turner et al. (9).

In our studies, we occasionally observed small changes in p125 FAK phosphorylation in response to Ang II or PDGF; however, these variations were minor relative to the large increase in CADTK Tyr(P) observed in these samples. The cause for the discrepancies between our results and the 4-fold difference of Polte et al. is difficult to ascertain and may represent differences in experimental conditions or antisera used in these studies. In one study, the antisera used to study p125 FAK was raised against the COOH-terminal 150 amino acid residues of mouse p125 FAK (36), an area of high sequence homology to rat CADTK. Thus, some of the increased phosphorylation attributed to p125 FAK may be a consequence of antibody cross-precipitation to both p125 FAK and CADTK, whereas the 50% increase observed by Turner et al. (9) is probably within the range of experimental variation.

Ang II and PDGF have also been reported to increase the tyrosine phosphorylation of paxillin, presumably through the regulation of p125 FAK in smooth muscle cells (9, 33, 34). Although paxillin is a substrate for p125 FAK phosphorylation in vitro, the mechanism by which this and PKC increase CADTK activity in vivo remains to be elucidated; our results suggest that this is mediated through a cytoskeleton-dependent mechanism (see below).

**DISCUSSION**

FIG. 6. The effect of cytochalasin D on CADTK Tyr(P). A, RSMC were prepared as described above and treated with carrier (Me2SO, lanes 1–5) or cytochalasin D (10 \( \mu \)M, 10 min, lanes 6–10) prior to the addition of the following stimuli. Lanes 1 and 6, no addition; lanes 2 and 7, 1.5 nM PDGF (10 min); lanes 3 and 8, 1 \( \mu \)M Ang II (3 min); lanes 4 and 9, 100 nM TPA (10 min); lanes 5 and 10, pervanadate (100 \( \mu \)M, 10 min). CADTK was immunoprecipitated, and the amount of CADTK Tyr(P) was determined by immunoblotting as above. B, the remainder of the lysates from A were immunoprecipitated and immunoblotted, and the amount of CADTK was visualized by ECL as described earlier.

FIG. 7. Immunolocalization of CADTK in RSMC. RSMC were grown on glass coverslips, serum-starved, fixed, and permeabilized for immunostaining as described under “Experimental Procedures.” CADTK protein was detected by immunostaining with a fluorescein isothiocyanate analog coupled to a \( \alpha \)-rabbit secondary antibody. A, localization of CADTK to focal adhesions and actin stress fibers in RSMC (\( \times \)60 magnification). B and C, co-localization of CADTK on actin filaments. B, RSMC were stained for CADTK (\( \times \)100 magnification) as described in A. C, RSMC were co-stained for CADTK as described in A and were stained for actin with rhodamine-conjugated phalloidin (2 \( \mu \)g/ml) for 30 min at room temperature (\( \times \)100 magnification).

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\(^a\) P. Diliberto, unpublished observations.

\(^b\) Y. He, unpublished observations.
Regulation of CADTK in Vascular Smooth Muscle Cells

vitro, in a previous report the Ang II-stimulated increase in paxillin phosphorylation did not correlate well with the effects of these stimuli on p125FAK (9), suggesting that additional kinases might contribute to this event. Recently, CADTK has been shown to co-immunoprecipitate with and to preferentially phosphorylate paxillin in some cells (26, 27). In RSMC, we also find that CADTK and paxillin co-immunoprecipitate and find that the agonist-stimulated increase in paxillin phosphorylation correlates well with the effects of these stimuli on CADTK phosphorylation. Paxillin was observed to be weakly phosphorylated in unstimulated cells, suggesting that p125FAK contributes to the basal levels of paxillin phosphorylation. In comparison, the stimulated increase in paxillin phosphorylation paralleled the increase in CADTK activity and was inhibited by EGTA, which inhibited the agonist-stimulated CADTK activity but not p125FAK. Prior to the discovery of CADTK, Leduc and Meloche (33) also reported that paxillin was phosphorylated in a calcium-dependent manner in smooth muscle cells. Our studies are consistent with these earlier studies and suggest that CADTK contributes to the stimulated (calcium- and PKC-dependent) increase in paxillin Tyr(P), whereas the basal levels of paxillin phosphorylation may be mediated by p125FAK in adherent RSMC. However, although the increase in paxillin Tyr(P) correlates with an increase in CADTK activity, the possibility that this phosphorylation is catalyzed by a CADTK-associated kinase cannot be excluded. Both Src and Src family members tyrosine kinases (Lck, Fyn) directly associate with CADTK (Pyk-2/RAFTK (18, 19)) and could potentially contribute to this event.

We also provide evidence illustrating the importance of CADTK cytoskeletal interactions. Agonist-dependent activation of CADTK was blocked by disruption of the actin cytoskeleton with cytochalasin D, consistent with other reports indicating that, like p125FAK, activation of CADTK is dependent on the integrity of the actin cytoskeleton (24, 25, 27). In contrast, the pervanadate-stimulated CADTK phosphorylation was not inhibited by cytochalasin D, suggesting that this treatment disrupted the factor (or factors) required for agonist-dependent activation of CADTK. Although previous studies have not clearly established the intracellular localization of CADTK, our studies now suggest the importance of actin filament association. Using transient overexpression systems (COS), Sasaki and co-workers (15) had reported that CADTK/Caβ3 was found in areas of cell-cell contact. Instead, Li et al. (19) suggested co-localization of RAFTK/CADTK with vinculin and focal adhesion-like structures in COS or CMK megakaryocytes. Our co-localization of RAFTK/CADTK with vinculin and focal adhesions in these cells.5 It is important to emphasize that we examined the immunofluorescence of endogenous CADTK in RSMC rather than in cells over-expressing artificially high levels of CADTK. We also acknowledge Bunei Li and Ruth Dy for excellent technical assistance.

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REFERENCES

1. Ross, R. (1995) Nature 362, 801–809
2. Lee, R. M., Owens, G. K., Scott-Burden, T., Head, R. J., Mulvany, M. J. & Schiffrin, E. L. (1995) Can. J. Phys. Pharm. 73, 574–584
3. Bornfeldt, K. E., Rains, E. W., Graves, L. M., Skinner, M. P., Krebs, E. G. & Ross, R. (1995) Ann. N. Y. Acad. Sci. 766, 416–430
4. Claesson-Welsh, L. (1994) J. Biol. Chem. 269, 32023–32026
5. Naftilan, A. J. (1994) Curr. Opin. Nephrol. Hypertens. 3, 218–227
6. Huckle, W. R. & Earp, H. S. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 338–342
7. Ishida, M., Marrelli, M. B., Schieffer, B., Itriha, T., Bernstein, K. E. & Earp, H. S. (1993) J. Biol. Chem. 268, 31206–31213
8. Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Avraham, H. (1997) J. Biol. Chem. 272, 2184–2188
9. Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. & Parsons, J. T. (1994) Mol. Cell. Biol. 14, 1680–1688
10. Li, J., Avraham, H., Rogers, R. A., Raja, S. & Avraham, S. (1996) Blood 88, 417–428
11. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A. & Schlessinger, J. (1996) Nature 383, 547–50
12. Tokiwa, G., Dikic, I., Lev, S. & Schlessinger, J. (1996) Science 273, 792–794
13. Graves, L. M., He, Y., Lambert, J., Hunter, D., Li, X. & Earp, H. S. (1995) J. Biol. Chem. 270, 1920–1926
14. Higashimoto, K., Avraham, S., Raja, S. & Avraham, H. (1997) J. Biol. Chem. 272, 10941–10947
15. Astier, A., Avraham, H., Manie, S. N., Groopman, J., Canty, T., Avraham, M., Freeberg, T. (1996) J. Biol. Chem. 272, 228–232
16. L. M. Graves, unpublished observations.
26. Salgia, R., Avraham, S., Pisick, E., Li, J. L., Raja, S., Greenfield, E. A., Sattler, M., Avraham, H. & Griffin, J. D. (1996) J. Biol. Chem. 271, 31222–31226
27. Li, X. & Earp, H. S. (1997) J. Biol. Chem. 272, 14341–14348
28. Ross, R. & Kariya, B. (1980) Morphogenesis of Vascular Smooth Muscle Cells in Atherosclerosis and Cell Culture, pp. 69–91, American Physiological Society, Bethesda, MD
29. Earp, H. S., Huckle, W. R., Dawson, T. L., Li, X., Graves, L. M. & Dy, R. (1995) J. Biol. Chem. 270, 28440–28447
30. Diliberto, P. A., Gordon, G. W., Yu, C. L., Earp, H. S. & Herman, B. (1992) J. Biol. Chem. 267, 11888–11897
31. Parsons, J. T., Schaller, M. D., Hildebrand, J., Leu, T. H., Richardson, A. & Otey, C. (1994) J. Cell. Sci. (Suppl.) 18, 109–113
32. Roe, M. W., Hepler, J. R., Harden, T. K. & Herman, B. (1989) J. Cell. Physiol. 139, 100–108
33. Leduc, I. & Meloche, S. (1995) J. Biol. Chem. 270, 11367–11376
34. Abedi, H., Dawes, K. E. & Zachary, I. (1995) J. Biol. Chem. 270, 11367–11376
35. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. & Parsons, J. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5192–5196
36. Polte, T. R., Naftilan, A. J. & Hanks, S. K. (1994) J. Cell. Biochem. 55, 106–119
37. Duff, J. L., Berk, B. C. & Corson, M. A. (1992) Biochem. Biophys. Res. Commun. 188, 257–264
38. Bornfeldt, K. E., Raines, E. W., Nakano, T., Graves, L. M., Krebs, E. G. & Ross, R. (1994) J. Clin. Invest. 93, 1266–1274
39. Giasson, E. & Meloche, S. (1995) J. Biol. Chem. 270, 5225–5231
40. Xu, Q., Liu, Y., Gorospe, M., Udelsman, R. & Holbrook, N. J. (1996) J. Clin. Invest. 97, 508–514