Role of the Actin Cytoskeleton in G-Protein–Coupled Receptor Activation of PYK2 and Paxillin in Vascular Smooth Muscle

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Abstract—Dynamic remodeling of the actin cytoskeleton occurs during agonist-induced smooth muscle contraction. Tyrosine phosphorylation of the adaptor protein paxillin has been implicated in regulation of actin filament formation and force development. We have investigated the role of the actin cytoskeleton in noradrenaline (NA)-induced and endothelin (ET)-induced activation of the calcium-dependent nonreceptor tyrosine kinase PYK2 and subsequent phosphorylation of paxillin in rat small mesenteric arteries. NA and ET induced a rapid and prolonged activation of PYK2, as shown by increased phosphorylation at Y402 and Y881, and a concomitant association of the kinase with a Triton X-100 insoluble membrane (cytoskeleton) compartment. Both agonists also increased phosphorylation of paxillin at Y31 and Y118 with a similar time course as PYK2 phosphorylation, and induced its association with the same membrane compartment as PYK2. Treatment of arteries with cytochalasin D disrupted stress fibers and inhibited NA-induced and ET-induced force in a myosin light chain 20 phosphorylation independent and reversible manner. However, cytochalasin D treatment had no effect on NA-induced and ET-induced phosphorylation of either PYK2 or paxillin but did prevent their association with the TritonX-100 insoluble membrane compartment. These results show that in mesenteric arteries an intact cytoskeleton and force development are not prerequisites for G-protein–coupled receptor–induced activation of PYK2 and paxillin, by tyrosine phosphorylation, in vascular tissue, but are necessary for the translocation of PYK2 and paxillin to the membrane. (Hypertension. 2005;46:93-99.)

Key Words: kinase ■ mesenteric arteries ■ phosphorylation

Small arteries adapt rapidly to changes in their environment. For instance, increased intraluminal pressure induces contraction and acute remodeling of the arterial wall allowing adaptation of vascular diameter and wall thickness to local flow and pressure.1 However, in pathological states, arterial tissue remodeling contributes to cardiovascular disorders. For example in hypertension, resistance arteries have increased wall thickness and reduced lumen diameter even when fully dilated.2 Recent evidence suggests that reorganization of the actin cytoskeleton is an important component of agonist-induced smooth muscle contraction.3 However, the signaling pathway involved in this response is not fully resolved. In airway and vascular smooth muscle tissues, tyrosine phosphorylation of the cytoskeleton-associated protein paxillin increases after stimulation with contractile agonists in parallel with force generation4–6 and its membrane association appears to be important for ACh-induced contraction,7,8 suggesting that a tyrosine kinase (TK)/paxillin/cytoskeleton pathway is an important component of smooth muscle contractility. However, whereas extracellular calcium is required for smooth muscle contraction, it is not essential for tyrosine phosphorylation of paxillin in response to ACh9 or endothelin,5 suggesting that contractility and paxillin phosphorylation are regulated by independent/parallel pathways in intact smooth muscle preparations.

PYK2 is a calcium-dependent nonreceptor (NR) TK related to FAK.10 In vascular smooth muscle cells, stimulation with angiotensin II increases tyrosine phosphorylation and kinase activity of PYK2 in a calcium and protein kinase C-dependent manner,11–13 suggesting that PYK2 activation is downstream of phosphoinositide signaling in smooth muscle. Additionally, there is evidence that the cytoskeleton is involved in the regulation of PYK2 activity. For instance, in nonstimulated cells PYK2 is localized throughout the cytoplasm, but on activation relocates to the actin cytoskeleton;11,12 additionally, inhibition of actin stress fiber formation inhibits the activation of PYK2 in response to angiotensin II in smooth muscle, endothelial cells, and epithelial cells,13,14,15 During agonist-stimulated contraction of smooth muscle tissue, actin polymerization occurs and is necessary for force development.16–18 However, it is unclear whether actin fiber organization and contractility are regulators of or regulated by NRTKs in smooth muscle tissues during G-protein–coupled receptor (GPCR) stimulation. Activated PYK2 asso-
ciates with paxillin and increases its tyrosine phosphorylation.\textsuperscript{19–21} Although the PYK2 phosphorylation sites on paxillin have not been identified, FAK in association with Src phosphorylates paxillin at tyrosine-31 (Y31) and Y118, creating 2 SH2-binding sites for Crk adaptor proteins,\textsuperscript{20,21} an event important in coordinating cell motility.\textsuperscript{22} Recently, it was shown that downregulation of the adaptor protein p130 Crk-associated substrate in strips of canine carotid artery reduced actin polymerization and force development in response to phenylephrine stimulation,\textsuperscript{23} further implicating paxillin-mediated reorganization of the actin cytoskeleton in smooth muscle contractility.

Our aims were to investigate whether GPCR-induced activation of PYK2 and paxillin were dependent on an intact actin cytoskeleton and force development in vascular tissue. We show that NA and ET activate PYK2 and paxillin, inducing their association with a cytoskeleton-enriched membrane fraction in intact rat mesenteric small arteries. Furthermore, disruption of actin filaments inhibited cytoskeleton association but not activation of PYK2 and paxillin in response to these agonists. Additionally, cytoskeleton disruption inhibited force development, but in an MLC20-independent manner. These results suggest that activation of PYK2 and paxillin is upstream of cytoskeletal rearrangements in vascular smooth muscle contraction.

### Materials and Methods

#### Preparation of Small Arteries

Adult Sprague Dawley rats (200 to 300 grams) were used for all experiments. All procedures performed were performed in accordance with institutional guidelines and the UK Animals (Scientific Procedures) Act of 1986. The mesentery was excised and kept in ice-cold physiological salt solution (PSS) until dissection.\textsuperscript{6,24} Mesenteric small arteries <400 μm internal diameter were cleaned of adjoining fat and connective tissues and equilibrated in culture medium (M199; GIBCO-BRL) for 45 minutes at 37°C before use.

#### Western Blotting

Proteins were extracted or subcellular fractions prepared from mesenteric arteries as described previously.\textsuperscript{5,24,25} Total tissue extracts (20 μg protein) or subcellular fractions were subjected to SDS-PAGE and processed for Western blot analysis by incubating with the appropriate phospho-specific antibody for 15 hours at 4°C. Signals were developed by horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Pierce, United Kingdom). After signal development, membranes were stripped and reprobed with the corresponding pan antibody. Band intensities were measured using densitometry and the phospho-antigen signal was corrected for total antigen.

**Figure 1.** Time-dependent phosphorylation of PYK2 by NA and ET. Arteries were treated with NA 15 μmol/L or ET 100 nmol/L for various time points up to 60 minutes and PYK2 phosphorylation measured as detailed in Materials and Methods. Y402PYK2 (A) and Y881PYK2 (B) after NA stimulation. Y402PYK2 (C) and Y881PYK2 (D) after ET stimulation. Upper panel, Representative membranes. Lower panel, Densitometric data of the phospho-antigen signal corrected for total antigen. Results are mean±SEM (n=3, *P<0.05 compared with 0 seconds).
Myosin Light Chain Phosphorylation
Myosin light chain (MLC) phosphorylation was measured according to the method of Kubota et al.26 Briefly, mesenteric small arteries were denatured in acetone containing 10% trichloroacetic acid (TCA) and 10 mmol/L dithiothreitol at −60°C, warmed to room temperature, and homogenized in 10% TCA containing 10 mmol/L dithiothreitol. Homogenates were washed with diethylether and protein extracted in 8 mol/L urea buffer. Proteins were subjected to urea/glycerol PAGE, transferred to nitrocellulose membrane, and probed with an antibody to MLC20.

Small Artery Contraction
Contractile responses were determined from segments of artery (~300 μm internal diameter) mounted in a pressure myograph as described.6,24 The artery segment was set to an intraluminal pressure of 70 mm Hg, an approximation of in vivo pressure and left to stabilize for 15 minutes. HEPES-buffered PSS pH 7.4 gassed with 5% CO2 in air was used for all incubations and vessel diameter was monitored continuously. The vessel was activated by 3 consecutive additions of 15 μmol/L NA (2.5 minutes and 5 to 10 minutes wash), then incubated for 60 minutes with 3 μmol/L cytochalasin D the response to NA 15 μmol/L 2.5 minutes was recorded before treatment, in the presence of cytochalasin D, and 60 minutes after washout. To test the effect of cytochalasin D on ET-induced contraction, vessels were incubated with inhibitor for 60 minutes before addition of 100 nmol/L ET. Control responses were obtained in arteries treated with vehicle (0.1% DMSO). The difference in protocol between the 2 agonists was required as ET-induced contraction is resistant to washout. Lumen diameter, measured 2 minutes after addition of agonist, was used as a measure of contractility.

Statistical Analysis
Data were analyzed by Student t test or repeated measures ANOVA with Dunnett correction for multiple comparisons using GraphPad Prism software. *P<0.05 was considered statistically significant. Many of the reagents used were obtained from sources previously described.5,6,24 Endothelin was purchased from Calbiochem, and Cytochalasin D and noradrenaline were from Sigma. Phospho-specific antibodies to PYK2 and paxillin were purchased from BioSource International; PYK2 (clone 11) and paxillin (clone 349) were from Transduction Laboratories. Horseradish peroxidase–conjugated secondary antibodies were from Jackson Laboratories. MLC20 polyclonal antibody was a gift from Dr K.E. Kamm, Dallas, Texas.

Results
NA and ET Stimulate the Tyrosine Phosphorylation of PYK2 and Paxillin
In rat mesenteric arteries, NA induces the tyrosine phosphorylation of paxillin.6 Paxillin contains 2 major sites of tyrosine phosphorylation Y31 and Y118, the phosphorylation of
which may be regulated by PYK2. PYK2 itself is activated by tyrosine phosphorylation at Y402 (activation site) and Y881 (Grb2 binding site). Using phospho-specific antibodies directed against these sites, we observed that basal tyrosine phosphorylation of PYK2 at Y402 and Y881 was low (Figure 1). However, stimulation with NA (15 μmol/L) and ET (100 nmol/L) increased their phosphorylation. This increase in PYK2 phosphorylation was rapid, detected within 40 seconds to 1 minute, and remained elevated above control for up to 20 minutes with both agonists and at both phosphorylation sites (Figure 1). Tyrosine phosphorylation of paxillin at Y31 and Y118 was also increased by NA and ET following a similar time course as PYK2, increasing within 30 seconds to 1 minute and returning to basal levels by 40 minutes (Figure 2).

**PYK2 and Paxillin Redistribute to a TritonX-100 Insoluble Membrane Fraction in Response to NA and ET**

Given that NA and ET activate both PYK2 and paxillin, we studied their redistribution after stimulation. Postnuclear homogenates were separated by centrifugation into cytosolic and membrane fractions. The membrane pellet was extracted with TritonX-100 to produce triton soluble and insoluble (actin cytoskeleton enriched) fractions. The fractions were immunoblotted with antibodies to actin, c-Src, PYK2, and paxillin. Actin was present mainly at the membrane (69%) and predominantly in the Triton-insoluble fraction; c-Src had a similar distribution, with 75% detected at the membrane again, mainly in the triton insoluble fraction (Figure 3A), showing this fraction is enriched in cytoskeletal proteins. In control tissues, PYK2 was predominantly cytosolic (75%), with the remaining 25% in the triton insoluble membrane compartment (Figure 3B). Paxillin was also predominantly cytosolic (75%), with the remaining 25% divided equally between triton soluble and insoluble fractions (Figure 3C). Stimulation with NA 15 μmol/L or ET 100 nmol/L for 5 minutes induced a redistribution of PYK2 and paxillin from the cytosol to the triton insoluble fraction (Figure 3B and 3C).

**Disruption of the Actin Cytoskeleton with Cytochalasin D Inhibits Contraction Independent of MLC20 Phosphorylation**

Both NA and ET are powerful vasoconstrictors, and we wished to determine whether a viable actin cytoskeleton was necessary for the contraction induced by these 2 agonists. We used cytochalasin D, which caps the barbed end of actin filaments promoting depolymerization of F-actin and therefore disruption of the cytoskeleton. Treatment of small arteries with 3 μmol/L cytochalasin D for 60 minutes reduced the levels of filamentous actin in the triton insoluble fraction (ratio [soluble/insoluble] for nontreated, 0.86±0.01; ratio for cytochalasin D, 1.37±0.03; P<0.01; n=3) and inhibited (~80% reduction) contraction to NA and ET (Figure 4A and 4B). The effect on contractility was reversible, demonstrating that cytochalasin D treatment was not toxic to the tissue. To show that cytochalasin D did not affect contractility through a nonspecific effect, we measured MLC20 phosphorylation, which is necessary for contraction. Western blot analysis shows that cytochalasin D treatment did not reduce NA-induced or ET-induced MLC20 phosphorylation (Figure 4C), either in the initial or in the sustained phase of contraction.
Disruption of the Actin Cytoskeleton With Cytochalasin D Inhibits Agonist-Induced Membrane Association of PYK2 and Paxillin

We have shown that activation of PYK2 and paxillin is associated with their relocation to the Triton insoluble, ie, cytoskeletal fraction. Given that cytochalasin D disrupted actin filaments and significantly inhibited NA-induced and ET-induced contraction, we studied the distribution profile of PYK2 and paxillin in response to the same 2 agonists. Treatment of small arteries under the same conditions that inhibited contraction (3 μmol/L cytochalasin D, 60 minutes) did not substantially alter the subcellular distribution of PYK2 or paxillin in control tissue but did completely block their movement to the membrane in response to NA and ET (Figure 5).

Disruption of the Actin Cytoskeleton Does Not Inhibit Tyrosine Phosphorylation of PYK2 or Paxillin

To investigate whether disruption of the actin cytoskeleton and inhibition of contraction affected activation of PYK2 or paxillin, arteries were treated with vehicle (DMSO 0.03%) or cytochalasin D before stimulation with NA and ET. There was no inhibition of PYK2 phosphorylation at Y402 and Y881 or paxillin at Y31 and Y118 in the presence of cytochalasin D (Figure 6) when compared with the vehicle control (data not shown).

Discussion

Contractile stimuli have been shown to induce the formation of filamentous actin in vascular and airway smooth muscle tissues, as well as in cultured smooth muscle cells, indicating that reorganization of the actin cytoskeleton may be an important component of smooth muscle contraction. Approximately 25% of smooth muscle actin are in the monomeric form and exist in a dynamic...
equilibrium with filamentous actin (F-actin). However, after stimulation, this equilibrium alters to favor an increase in F-actin associated with cytoskeletal structures. Recent evidence implicates the adaptor protein paxillin as playing a role in cytoskeletal remodeling during ACh-induced contraction, and both tyrosine phosphorylation and membrane-association of paxillin are necessary for full-force development to this agonist. Here we show that 2 other contractile agonists NA and ET activate and induce association of paxillin with a cytoskeleton-enriched membrane fraction in vascular tissue, suggesting that activation of paxillin is a common pathway used by GPCR agonists to regulate contraction. In response to NA and ET, we observed increased phosphorylation of paxillin at residues Y31 and Y118, which are phosphorylation sites critical for actin reorganization in ACh-stimulated airway smooth muscle, implicating activation of NRTKs as upstream regulators of paxillin function. Paxillin itself is a substrate for the NRTK PYK2, and NA and ET both increased PYK2 phosphorylation at residues Y402 and Y881 with a time course consistent with involvement in the sustained phase of smooth muscle contraction. Phosphorylation of these residues reflects increased kinase activity, indicating that PYK2 could be involved in paxillin phosphorylation in response to GPCR signals. The close correlation of the time courses of PYK2 and paxillin phosphorylation provides circumstantial evidence for such a possibility. Furthermore, NA and ET induced translocation of PYK2 from the cytosol to the cytoskeleton-enriched membrane fraction that also contained actin and c-Src, known cytoskeleton-associated proteins, suggesting that PYK2 and paxillin are involved in signaling to the cytoskeleton after NA and ET stimulation. In smooth muscle tissue, actin filaments are attached to the plasma membrane at dense plaques and our observation that the PYK2-enriched fraction also contained the known focal adhesion proteins, Src and paxillin, implies that PYK2 was associating with dense plaques. This suggests that PYK2 couples GPCR signals to the reorganization of the cytoskeleton in response to acute signals. However, further studies interfering selectively with PYK2 activation/ function are required to substantiate this.

To investigate further the relationship between PYK2 and paxillin activation and the actin cytoskeleton, we used cytochalasin D to disrupt actin stress fiber formation. Treatment of small arteries with cytochalasin D increased the pool of soluble (monomeric) actin within the tissue indicating disruption of actin filaments. This was accompanied by a substantial reduction in force development in response to both NA and ET without a reduction in MLC20 phosphorylation, demonstrating that cytochalasin D was not inhibiting the phosphoinositide/calcium/MLCK pathway of contraction, nor was cytochalasin D toxic, because its subsequent removal restored full contractile function. These data are consistent with previous studies demonstrating that inhibition of force development as a consequence of disrupting the actin cytoskeleton is independent of MLC20 phosphorylation. Cytochalasin D treatment prevented the translocation of PYK2 and paxillin to the cytoskeleton-enriched membrane fraction in response to NA and ET, providing further evidence that these signaling molecules are associating with the cytoskeleton. In contrast, cytochalasin D treatment had no effect on NA-induced and ET-induced PYK2 or paxillin tyrosine phosphorylation. These data are in agreement with a recent study in cardiac myocytes in which tyrosine phosphorylation of PYK2 induced by mechanical stress was not inhibited by cytochalasin D, but are in contrast to other reports that disruption of the cytoskeleton inhibits phosphorylation of FAK, PYK2, and paxillin in cells in culture. The reason for this difference is unclear but may reflect differences in the signaling pathways, leading to PYK2 activation in tissues compared with cells. Accordingly, in mesenteric arteries, an intact cytoskeleton or force development are not prerequisites for GPCR-associated PYK2 activation and subsequent tyrosine phosphorylation of paxillin in vascular tissue but are necessary for the translocation of PYK2 and paxillin to the membrane. However, unlike the study in tracheal smooth muscle in which expression of nonphosphorylatable mutant of paxillin inhibited tension development without affecting MLC20 phosphorylation, our results do not suggest a role for paxillin phosphorylation per se in the contractile response to NA or ET. Although this could be
caused by differences in tissues and/or agonists used in the studies, it may well reflect the specialized functions of the different smooth muscle tissues.

**Perspectives**

Our data indicate that NA and ET activate PYK2 and paxillin and induce their redistribution to an actin cytoskeleton-enriched membrane compartment with a time course consistent with contraction. However, although an intact actin cytoskeleton and force development were required for membrane association, they were not a prerequisite for tyrosine phosphorylation of PYK2 and paxillin in response to GPCR agonists.

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**References**

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