Calcium- and Protein Kinase C–Dependent Activation of the Tyrosine Kinase PYK2 by Angiotensin II in Vascular Smooth Muscle

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Abstract—Angiotensin II (Ang II) induces vascular smooth muscle cell (VSMC) growth by activating Gq-protein–coupled AT1 receptors, which leads to elevation of cytosolic Ca2+ ([Ca2+]i) and activation of protein kinase C (PKC) and mitogen-activated protein kinases. To assess the link between these Ang II–induced signaling events, we examined the effect of Ang II on the proline-rich tyrosine kinase (PYK2), previously found to be activated by a variety of stimuli that increase [Ca2+]i, or activate PKC. PYK2 distribution was demonstrated in rat aortic tissue and in cultured VSMC by immunohistochemistry, revealing a cytosolic distribution distinct from smooth muscle α-actin, focal adhesion kinase, or paxillin. The involvement of PYK2 in Ang II signaling was measured by immunoprecipitation and immune complex kinase assays. Treatment of quiescent VSMC with Ang II resulted in a concentration- and time-dependent increase in PYK2 tyrosine phosphorylation and kinase activity in PYK2 immunoprecipitates. PYK2 phosphorylation was inhibited by AT1 receptor blockade and was attenuated by downregulation of PKC or the chelation of [Ca2+]i. Treatment with either phorbol ester or Ca2+ ionophore also increased PYK2 phosphorylation, suggesting that PKC activation and/or increased [Ca2+]i are both necessary and sufficient to activate PYK2. Activation of PYK2 by Ang II was also associated with increased PYK2-src complex formation, suggesting that PYK2 activation represents a potential link between Ang II-stimulated [Ca2+]i and PKC activation with downstream signaling events such as mitogen-activated protein kinase activation involved in the regulation of VSMC growth.

Key Words: PYK2 ▪ vascular smooth muscle ▪ angiotensin II ▪ protein kinase C ▪ Ca2+

Angiotensin II (Ang II) plays an important role in regulating systemic blood pressure through the modulation of vascular smooth muscle contraction and blood volume. Ang II also acts as a growth factor for vascular smooth muscle cells (VSMC), inducing VSMC hypertrophy and/or hyperplasia in vitro1–3 and modulating the expression of immediate response genes such as c-fos4 and c-myc.5 A role of Ang II in VSMC growth and tissue remodeling has also been shown in animal models of hypertension, atherosclerosis, and restenosis.6–8

Ang II binds to at least 2 high-affinity receptors, designated AT1 and AT2.9 All of the physiological and growth effects of Ang II in VSMC are mediated through the AT1 receptor based on effects of specific pharmacological inhibitors.10 This receptor has been cloned and contains the structural features of a 7-transmembrane domain, heterotrimeric G-protein–coupled receptor.11

It has become clear that many intracellular signaling events mediated by AT1 receptors are similar to those involved in signaling pathways activated by growth factor receptor tyrosine kinases.12 In fact, activation of AT1 receptors leads to an increase in tyrosine phosphorylation of several proteins such as Shc13, PLCγ,14 and STAT1,15 and stimulates several nonreceptor tyrosine kinases including focal adhesion kinase (pp125FAK),16 JAK2,17 and src.18 Furthermore, mitogen-activated protein (MAP) kinases, which are important downstream effectors in growth factor–induced changes in gene expression, have also been proposed to play a critical role in mediating Ang II–induced VSMC growth.18,19 Activation of MAP kinases by Ang II requires increased [Ca2+]i, and/or activation of protein kinase C (PKC).19–22 but the intermediate steps between increased [Ca2+]i/PKC activation and activation of MAP kinase have not been identified.

An attractive candidate for intermediate signaling is the recently identified proline-rich tyrosine kinase (PYK2) also known as RAFTK, CAKβ, or CADTK.23–26 PYK2 is a member of a family of nonreceptor tyrosine kinases that also includes pp125FAK23,25 Both pp125FAK and PYK2 have been implicated as important integrating molecules in signal transduction cascades. It has been suggested that PYK2 plays a role in signaling in a variety of cell types, including PC12 cells (a pheochromocytoma-derived cell line),24,25 rat liver...
epithelial cells, T and B lymphocytes, astrocytes, hippocampal neurons, megakaryocytes, and recently, VSMC. In PC12 cells, it has been found that PYK2 is activated by phosphorylation on one or more tyrosine residues in response to stimuli, such as activation of G-protein-coupled receptors that elevate [Ca\(^{2+}\)]\(_i\), or activate PKC. Once phosphorylated, PYK2 can recruit other signal-transducing molecules with SH2 domains such as the tyrosine kinase src or the adapter molecules Shc and Grb2. Complex formation between PYK2 and src or Grb2 may ultimately lead to MAP kinase activation.

In the present study, we show that PYK2 is expressed in VSMC with an intracellular distribution distinct from smooth muscle \(\alpha\)-actin (SM \(\alpha\)-actin), pp125\(^{FAK}\), or paxillin. Ang II induces PYK2 phosphorylation in these cells in a concentration- and time-dependent manner that requires an increase in [Ca\(^{2+}\)]\(_i\) and/or activation of PKC. Furthermore, PYK2 activation is associated with the formation of a complex between PYK2 and the nonreceptor tyrosine kinase, src. A preliminary report has been published.

### Materials and Methods

**Materials**

An anti-PYK2 monoclonal antibody derived from the C-terminal portion of rat PYK2 (amino acids 833 to 997), anti-pp125\(^{FAK}\), anti-paxillin, isofom-specific anti-PKC antibodies, and polyclonal anti-phosphotyrosine (pTyr) antibody were obtained from Transduction Laboratories. A–Sepharose, protein G–agarose, and Ang II were from Sigma Technology. PMA, BAPTA-AM, chelerythrine chloride, and phorbol dibutyrate (PDBU) were from Alexis Laboratories. Protein samples were stored at \(-80^\circ\)C until use.

**Cell Culture**

Smooth muscle cells were isolated from 10- to 12-week-old male Sprague-Dawley rat thoracic aortae by enzymatic digestion as previously described. VSMC (at passages 3 to 8) were cultured in 10% calf serum-DMEM and were growth-arrested for 24 hours in serum-free DMEM before the start of the experiment.

**Western Blotting**

Rat aortic tissue samples were prepared by homogenization in lysis buffer containing 150 mmol/L NaCl, 1.2 mmol/L MgCl\(_2\), 1 mmol/L EGTA, 1 mmol/L NaVO\(_4\), 10 mmol/L Na pyrophosphate, 100 mmol/L NaF, 50 mmol/L HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 10 \(\mu\)g/mL leupeptin, 10 \(\mu\)mol/L aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentrations were assessed with use of a bichinonic acid assay (Pierce) and equal amounts of protein (50 \(\mu\)g) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond, Amersham). PYK2 and pp125\(^{FAK}\) were detected by Western blot analysis with use of a 1:1000 dilution of anti-PYK2 or anti-pp125\(^{FAK}\) antibodies, and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (BioRad). Bands were visualized by enhanced chemiluminescence (ECL, Amersham), and those corresponding to PYK2 were quantified by laser densitometry.

**Immunoprecipitation**

Growth-arrested VSMC were treated with Ang II as indicated in each experiment, rinsed with cold PBS, then lysed in ice-cold lysis buffer with 10% glycerol. PYK2 phosphorylation was examined by immunoprecipitation of 500 \(\mu\)g of cell lysate protein with monoclonal anti-PYK2 or polyclonal anti-p-Tyr antibodies overnight at 4°C. Immune complexes were collected by incubation with protein G–Sepharose or protein A–agarose beads for 2 hours at 4°C. The beads were centrifuged, washed in buffer containing 150 mmol/L NaCl, 1.2 mmol/L MgCl\(_2\), 1 mmol/L EGTA, 1 mmol/L NaVO\(_4\), 10 mmol/L Na pyrophosphate, 100 mmol/L NaF, 50 mmol/L HEPES, pH 7.4, 1% Triton X-100, 10 \(\mu\)g/mL leupeptin, 10 \(\mu\)g/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride and resuspended in Laemmli sample buffer. Immunoprecipitates were separated with use of SDS-PAGE and PYK2 phosphorylation was detected by immunoblotting as described above.

**PYK2 Immune Complex Kinase Assay**

Tyrosine phosphorylation of exogenous substrate by PYK2 immune complexes was performed as previously described by Sasaki et al. PYK2 was immunoprecipitated with monoclonal antibody anti-PYK2 from growth arrested VSMC treated with 100 mmol/L Ang II or vehicle for the indicated times. The immunoprecipitates were washed twice with wash buffer, once with 20 mmol/L Tris (pH 7.4), 0.5 mmol/L LiCl, once with 20 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, once with kinase buffer A (20 mmol/L Tris [pH 7.4], 4 mmol/L MgCl\(_2\), 1 mmol/L dithiothreitol), and then incubated with 30 \(\mu\)L of kinase buffer A containing 5 \(\mu\)g poly(Glu-Tyr), 1.5 \(\mu\)mol/L unlabeled ATP, 10 \(\mu\)Ci [\(\gamma\)\(^{32}\)P]ATP (3000 Ci/mmol, Amersham), and 5 mmol/L MgCl\(_2\) for 15 minutes at 20°C. The reactions were stopped by SDS sample buffer, resolved on 15% SDS-PAGE, analyzed by autoradiography, and quantified by laser densitometry. The data are presented as kinase activity relative to vehicle-treated cells. The accuracy of this method of quantification was confirmed by excising the area of the gel corresponding to poly(Glu-Tyr) and then measuring the radioactivity by liquid scintillation counting.

**PYK2 Phosphorylation in Immunoprecipitates**

Growth-arrested VSMC were treated with 100 mmol/L Ang II at different times. Cell lysates were immunoprecipitated with monoclonal anti-PYK2 antibodies and immunoprecipitates were washed twice with Triton-only lysis buffer, twice with HNTG buffer (20 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol), and twice with 20 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, once with kinase buffer A (20 mmol/L Tris [pH 7.4], 4 mmol/L MgCl\(_2\), 1 mmol/L dithiothreitol). To initiate kinase reactions, the excess buffer was removed, the reactions were stopped by SDS sample buffer, resolved on 15% SDS-PAGE, analyzed by autoradiography, and quantified by laser densitometry.

**Immunolabeling**

Cultured VSMC were fixed in cold ethanol for 10 minutes (for SM \(\alpha\)-actin and PYK2 immunolabeling) or in 4% paraformaldehyde for 20 minutes (for paxillin and pp125\(^{FAK}\) immunolabeling). After 2 washes, cells fixed with paraformaldehyde were treated with 0.1% Triton X-100 for 10 minutes and washed twice in PBS before immunolabeling. The aortic tissue preparation and immunolabeling conditions were performed as previously described. The antibodies used for immunolabeling were monoclonal anti-SM \(\alpha\)-actin, anti-PYK2, anti-pp125\(^{FAK}\), or anti-paxillin at

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dilutions of 1/50, 1/20, 1/50, and 1/500, respectively. Sections were mounted and observed with a Dialux microscope (Leica) equipped with epifluorescence optics.

Measurement of $[\text{Ca}^{2+}]_i$

Cells were loaded with fura-2 by incubating for 60 minutes with the acetoxymethyl ester form (fura-2-AM, 2 μmol/L) in modified Kreb’s buffer (containing in mmol/L: NaCl 135, KCl 5.9, CaCl₂ 1.5, MgCl₂ 1.2, HEPES 11.6, and D-glucose 11.5) supplemented with 0.1% BSA and 0.2% Pluronic F127. The cells were then washed twice and incubated for 60 minutes in modified Kreb’s buffer before measurement of fluorescence in a Perkin-Elmer LS50B fluorescence spectrophotometer. The coverslip was inserted into a 4.5-mL optical methacrylate cuvette on a 30° angle to the light beam. The solution bathing the cells was changed by perfusing fresh solution from gravity-fed reservoirs into the bottom of the cuvette while aspirating continuously from just above the coverslip. At the perfusion rates used (5 to 10 mL/min), the half-time for mixing in the cuvette was ≈20 seconds and complete exchange occurred within 50 seconds. The cells were excited alternately with 340 and 380 nm light every 0.02 seconds with a rotating filter wheel in the path of the excitation light. To correct for background fluorescence, the cells were treated for at least 10 minutes with ionomycin (1 μmol/L) and MnCl₂ (6 mmol/L) in Ca²⁺-free Kreb’s buffer to quench the fura-2 fluorescence. The remaining fluorescence at each wavelength was then subtracted from the experimental traces. An integrated ratio ($\frac{I_{340}}{I_{380}}$) of the light emitted at 510 nm was then determined at 0.5-second intervals.

Data Analysis

Concentration-response curves for Ang II were fit by nonlinear regression with use of PRISM software (GraphPad). All results were expressed as mean±SEM. One-way repeated-measures ANOVA (followed by Dunnett’s test or Bonferroni’s t test for comparisons among multiple groups) or a 2-tailed, paired Student t test were used for statistical comparisons. Differences among means were considered significant at $P<0.05$. Data were analyzed with use of InStat Statistical Software (GraphPad).

Results

PYK2 Expression in Vascular Smooth Muscle

PYK2 was detected by Western blotting in homogenates of rat aortic tissue and cell lysates from cultured aortic VSMC with use of 2 different anti-PYK2 antibodies. Both monoclonal (Figure 1A) and polyclonal (Figure 1B) antibodies recognized a single band of ≈112 kDa that was also found in PC12 cells but did not cross-react with the closely related pp125FAK (as determined by the lack of immunoreactivity with a ≈125-kDa protein). Re-probing of the blot shown in Figure 1A with a monoclonal anti-pp125FAK antibody showed a single band of apparent molecular weight of 125 kDa (Figure 1C).

Distribution of PYK2 in Vascular Smooth Muscle

The distribution of PYK2 in VSMC was examined by immunolabeling of rat aortic tissue and cultured VSMC with use of monoclonal antibodies directed against SM α-actin, PYK2, pp125FAK, and paxillin (Figure 2). In the aorta, SM α-actin labeling was homogeneously distributed throughout the VSMC layers of the media (Figure 2A). The pattern of PYK2 staining was similar to that of SM α-actin, although with less labeling intensity (Figure 2B). In cultured VSMC, SM α-actin labeling identified stress fibers (Figure 2D) and PYK2 immunolabeling was homogeneously distributed throughout the cytoplasm but undetectable in the nucleus (Figure 2E). This PYK2 labeling in VSMC was distinct from those of pp125FAK or paxillin which localized to the focal adhesion contacts (Figure 2F and 2H). PYK2 immunolabeling in PC12 cells (used as a positive control) was also homogeneously distributed throughout the cytoplasm (Figure 2I). The specificity of the PYK2 antibody was confirmed with anti-PYK2 antibodies preadsorbed with the PYK2 antigen peptide. No labeling was detected in either aortic tissue
Ang II Induces Rapid Tyrosine Phosphorylation of PYK2

Activation of PYK2 requires phosphorylation of tyrosine residue 402. In Figure 3A, we examined the concentration dependence for Ang II–induced tyrosine phosphorylation of PYK2. Quiescent VSMC were treated with 10^{-11} to 10^{-3} mol/L Ang II for 5 minutes, VSMC lysates were immunoprecipitated with monoclonal anti-PYK2 antibodies, and phosphorylated PYK2 was measured in the immunoprecipitate by Western blot analysis with monoclonal anti-pTyr antibodies (Figure 3A). Treatment with Ang II caused a rapid and sustained increase in PYK2 phosphorylation. Activation was detected at 0.5 minutes (2.1±0.1-fold versus control), was maximal at 5 minutes (4.8±0.9-fold versus control), and returned toward basal levels at 60 minutes (1.5±0.1-fold versus control). Nearly identical results were observed when phosphorylated PYK2 was immunoprecipitated with polyclonal anti-pTyr antibodies and PYK2 was detected in immunoblots with a monoclonal anti-PYK2 antibody (Figure 3C). PYK2 tyrosine phosphorylation was observed as early as 0.5 minutes (6±0.5-fold versus control), reached a maximum at 5 minutes (15±5-fold versus control), and remained phosphorylated at 60 minutes (2.6±0.3-fold versus control). Thus, both methods yielded a qualitatively similar time-course profile for PYK2 phosphorylation by Ang II. The difference in the magnitude observed between the 2 methods is unclear, but
may be caused by differences in the affinities of the antibodies used to immunoprecipitate PYK2 or for immunoblotting.

**Phosphorylation of PYK2 Is Associated With Formation of PYK2-src Complex**

Activation of src, tyrosine phosphorylation of Shc, and the formation of a Shc-Grb2 complex are all associated with Ang II–induced signaling in VSMC.\(^{13,17,38}\) To determine whether PYK2 phosphorylation can lead to the formation of these active signaling complexes within VSMC, cell lysates were immunoprecipitated with anti-PYK2 antibodies and analyzed by Western blot with anti-src polyclonal antibodies (Figure 4). In unstimulated cells, little association between PYK2 and src was observed. Stimulation with Ang II increased PYK2-src complex formation (Figure 4) which returned to baseline at 30 minutes. This antibody is specific for src, because it

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**Figure 3.** Ang II activates PYK2 tyrosine phosphorylation in VSMC. A, Concentration dependence for Ang II. VSMC were treated with \(10^{-11}\) to \(10^{-5}\) mol/L Ang II for 5 minutes. Cell lysates were immunoprecipitated with a monoclonal anti-PYK2 antibody, then immunoblotted with an anti-pTyr monoclonal antibody. The left upper panel is a representative immunoblot showing a concentration-dependent increase in phosphotyrosine content of PYK2 in response to Ang II. The lower panel is the same blot reprobed with anti-PYK2 showing equal amounts of PYK2 in each lane. The right lower panel summarizes quantitative results from densitometric analysis of 3 blots. B, Time course for Ang II. VSMC were treated with 100 nmol/L Ang II for 0 to 60 minutes, lysates were immunoprecipitated with a monoclonal anti-PYK2 antibody, and tyrosine phosphorylation of PYK2 was determined by Western blotting with a monoclonal anti-pTyr antibody. The panel on the right summarizes quantitative results from densitometric analysis of 4 blots (mean±SEM). Data are presented as a fold increase above control (ctrl) that was set at 1. *\(P<0.02\), **\(P<0.001\) vs control. C, VSMC were treated with 100 nmol/L Ang II for 0 to 60 minutes and immunoprecipitated with a polyclonal anti-pTyr antibody, and phosphorylated PYK2 was determined by Western blotting with a monoclonal anti-PYK2 antibody. The panel on the right summarizes quantitative results from densitometric analysis of 6 blots (mean±SEM). Data are presented as a fold increase above control (Ctrl), which was set at 1. *\(P<0.02\), **\(P<0.001\) vs control.
detected a single band of \( \approx 60 \text{kDa} \) by Western blot analysis of VSMC lysates that was also detected with a monoclonal anti-src antibody (data not shown).

It has been shown previously that the association of PYK2 with src requires PYK2 kinase activity and autophosphorylation of tyrosine residue 402. 27 Therefore, we determined whether the interaction of PYK2 and src induced by Ang II correlated with an increase in kinase activity. Cell lysates from control- and Ang II-treated cells were immunoprecipitated with anti-PYK2 monoclonal antibodies and Western blot analysis was performed with polyclonal anti-src antibodies. The blot is representative of 4 experiments.

Ang II Induced PYK2 Phosphorylation Through the AT₁ Receptor
To verify that the activation of PYK2 by Ang II was receptor-dependent and to determine which Ang II receptor subtype mediates this stimulation, the ability of Ang II to stimulate PYK2 phosphorylation was evaluated in the presence or absence of either losartan or PD123319 (10 \( \mu \text{mol/L} \)), specific AT₁ or AT₂ receptor antagonists, respectively. Treatment from control- and Ang II-treated cells were immunoprecipitated with an anti-PYK2 monoclonal antibody, and an in vitro kinase assay was performed in the presence of \([\gamma-^{32}\text{P}]\)ATP (10 \( \mu \text{Ci} \)) with use of poly(Glu-Tyr)\(^{4:1}\) as a substrate (Figure 5). Compared with basal levels, treatment with 100 nmol/L Ang II caused a rapid increase in kinase activity, with maximal activation at 1 to 2 minutes (18.3±1.8-fold to 17.7±2.5-fold versus control) that was sustained for 30 minutes and returned toward baseline at 60 minutes. Kinase activity was also measured by PYK2 phosphorylation. Exposure of cells to Ang II produced a similar increase in \(\gamma-^{32}\text{P}\)-incorporation into PYK2 (Figure 5B). A faint band was present at \(\approx 60 \text{kDa} \), consistent with the presence of src in the PYK2 immunoprecipitates.

Ang II Activates PYK2 in Vascular Smooth Muscle
ment of quiescent cells with 100 nmol/L Ang II for 5 minutes induced PYK2 phosphorylation which was completely inhibited by pretreatment with losartan. In contrast, PD123319 was without effect (Figure 6). These results demonstrate that Ang II-stimulated PYK2 phosphorylation is mediated via AT1 receptor activation.

Ang II–Induced PYK2 Phosphorylation in VSMC Requires an Increase in \([\text{Ca}^{2+}]_i\)

To determine the role of \([\text{Ca}^{2+}]_i\), elevation in mediating PYK2 phosphorylation in VSMC, we first treated the cells with 1 \(\mu\text{mol}/\text{L}\) ionomycin, a \(\text{Ca}^{2+}\) ionophore, for 0.5 to 30 minutes (Figure 7A). Treatment with ionomycin caused a rapid increase in PYK2 tyrosine phosphorylation that was detected at 0.5 minutes (4.1\(\pm\)0.9-fold increase versus control), reached maximum levels at 2 minutes (5.5\(\pm\)0.4-fold increase versus control), and decreased toward control by 30 minutes (1.5\(\pm\)0.5-fold increase versus control). We next explored the effect of cytosolic \(\text{Ca}^{2+}\) chelation on Ang II–induced PYK2 phosphorylation (Figure 7B). Cells were pretreated for 30 minutes with 50 \(\mu\text{mol}/\text{L}\) BAPTA, an intracellular \(\text{Ca}^{2+}\) chelator, before the addition of 100 nmol/L Ang II. We have previously shown that this concentration of BAPTA completely inhibits Ang II–induced \([\text{Ca}^{2+}]_i\) transients in VSMC. 20 Pretreatment of cells with BAPTA decreased Ang II–induced PYK2 phosphorylation at all times examined with maximum inhibition at 1 minute (82\(\pm\)5\% versus Ang II).

Ang II–Induced PYK2 Phosphorylation Requires PKC Activation

To determine the role of PKC activation in PYK2 phosphorylation, quiescent VSMC were incubated with 200 nmol/L PMA, a potent

Figure 6. Ang II induced PYK2 phosphorylation through AT1 receptor activation. Ang II receptor antagonists, losartan (10 \(\mu\text{mol}/\text{L}\), AT1-specific antagonist) and PD123319 (10 \(\mu\text{mol}/\text{L}\), AT2-specific antagonist), were added to growth-arrested VSMC 30 minutes before the addition of 100 nmol/L Ang II for 5 minutes. Phosphorylation of PYK2 was determined by immunoprecipitation of cell lysates with a polyclonal anti-pTyr antibody and Western blot analysis was performed with anti-PYK2 monoclonal antibodies. The immunoblot is representative of 3 different experiments.

Figure 7. Effect of cytosolic \(\text{Ca}^{2+}\) on PYK2 phosphorylation. A, VSMC were treated with the \(\text{Ca}^{2+}\) ionophore, ionomycin (1 \(\mu\text{mol}/\text{L}\)), for the indicated times. Cell lysates were immunoprecipitated with a polyclonal anti-pTyr antibody then immunoblotted with a monoclonal anti-PYK2 antibody. The panel on the right shows averaged results from densitometric analysis of 3 blots (mean\(\pm\)SEM). Data are presented as a fold increase above control (ctrl), which was set at 1. *P<0.01 vs control. B, Cell lysates were immunoprecipitated with an anti-pTyr antibody then immunoblotted with a monoclonal anti-PYK2 antibody. The panel on the right shows averaged results from densitometric analysis of 5 blots (mean\(\pm\)SEM). Data are presented as a fold increase above control (ctrl), which was set at 1. *P<0.01, **P<0.05 vs control; †P<0.01 vs Ang II–treated cells.
PKC activator, for 0.5 to 30 minutes (Figure 8A). In contrast to treatment with ionomycin, PYK2 phosphorylation was increased more slowly by treatment with PMA, reaching a maximum at 20 minutes (5.8 ± 0.5-fold versus control) and was sustained at 30 minutes. PMA treatment had no effect on intracellular Ca\(^{2+}\) in VSMC, compared with the rapid Ca\(^{2+}\) transient induced by Ang II (Figure 8B).

To further examine the requirement of PKC activation in Ang II–induced PYK2 phosphorylation, VSMC were pretreated with chelerythrine chloride, a potent PKC inhibitor that blocks PKC activation in VSMC and cardiac myocytes. VSMC were pretreated for 45 minutes with 5 μmol/L chelerythrine chloride before the addition of 100 nmol/L Ang II (Figure 8C). Ang II–induced PYK2 phosphorylation was significantly inhibited by pretreatment with chelerythrine chloride at all times studied.

Several PKC isoforms have been shown to activate MAP kinases in VSMC. To gain insight into the PKC isoforms required for Ang II–induced PYK2 phosphorylation, quiescent VSMC were pretreated for 24 hours with 1 μmol/L PDBU to downregulate PKC. In agreement with previous data, PDBU pretreatment for 24 hours caused PKC-α, -δ, -γ, and -ε downregulation, but had no detectable effect on the expression of PKC-β or the atypical PKC isoforms λ, η, and ζ (Figure 9A). PDBU pretreatment did not affect PYK2 expression (data not shown) but did cause a significant
decrease in Ang II–induced PYK2 phosphorylation (Figure 9B). A 44±5% decrease versus non-PDBU–treated cells was observed at 1 minute of Ang II treatment, and maximum inhibition was observed at 5 minutes (67±5% versus non-PDBU–treated cells) and was sustained for 20 minutes (63±2% versus non-PDBU–treated cells).

**Discussion**

The present study demonstrates the expression and distribution of PYK2 in both rat aortic tissue and cultured VSMC and characterizes its regulation by Ang II. Ang II stimulates PYK2 tyrosine phosphorylation in a concentration- and time-dependent manner. PYK2 phosphorylation is associated with an increase in its kinase activity and its interaction with src. An increase in [Ca^{2+}], and activation of PKC are both necessary and sufficient for PYK2 activation.

PYK2 expression was detected in intact aortae and cultured VSMC by Western blot analysis and immunocytochemistry. PYK2 immunolocalization in VSMC is distinct from SM α-actin, pp125 FAK, or paxillin demonstrating that PYK2 is not localized within focal adhesion contacts, but rather is homogeneously distributed throughout the cytoplasm. Our data also provide the first demonstration of PYK2 expression in intact aortae, and its codistribution with SM α-actin indicates that medial smooth muscle cells in vivo also express PYK2 (Figure 2). While the present study was in progress, 2 reports appeared describing immunocytochemical analyses of PYK2 localization in VSMC. Brinson et al reported colocalization of PYK2 with both actin and paxillin in focal adhesion contacts; whereas Zheng et al, in accord with our own findings, reported a cytosolic distribution of PYK2. The latter study provided additional evidence, using chimeric constructs, that the differential localization of PYK2 and pp125 FAK is caused by unique C-terminal domains of the 2 proteins.

In the present study, we show for the first time that PYK2 tyrosine phosphorylation by Ang II in VSMC is associated with an increase in its interaction with src (Figure 4). Ang II induced a rapid tyrosine phosphorylation of PYK2 that was associated with an increase in kinase activity, as determined by immune complex kinase assays. Because maximum kinase activity in PYK2 immunoprecipitates preceded significant
interaction between PYK2 and src (compare Figures 3 and 5), it is likely that PYK2 kinase activation and subsequent autophosphorylation result in complex formation between PYK2 and src. The different time courses for kinase activation and PYK2 phosphorylation, compared with increased PYK2-src complex formation, agree with previous studies demonstrating that autophosphorylation of tyrosine residue 402 in PYK2 is necessary for its activation and interaction with src in PC12 cells. A similar requirement for autophosphorylation was shown to be necessary for the interaction between pp125src and src. Activation of members of this kinase family may therefore result in a similar signaling paradigm whereby autophosphorylation on a tyrosine residue leads to complex formation with other signaling molecules via their SH2 domains. However, we cannot exclude the possibility that src or other kinases present in the PYK2 immunoprecipitate contribute to either phosphorylation of the exogenous substrate or of PYK2.

Since src has been shown to regulate MAP kinase signaling in VSMC, it is tempting to speculate that PYK2 activation in VSMC links AT1 receptor activation to src and the MAP kinase pathway. Activation of src has been shown to phosphorylate Shc and tyrosine-phosphorylated Shc associates with Grb2, leading to activation of the Ras-Raf-MAP kinase pathway. Studies by Lev et al and Dikic et al demonstrated that dominant negative mutants of PYK2 blocked the formation of the Shc-Grb2 signaling complex and, consequently, MAP kinase activation in PC12 cells. However, activation of different MAP kinases by PYK2 appears to be cell type specific. In PC12 cells, HEK-293 cells, and astrocytes, PYK2 activation is apparently required for activation of ERK1 and ERK2, but in rat liver epithelial cells, PYK2 activation appears to be coupled with c-Jun N-terminal kinase, but not with ERK1 and ERK2. In the present study, the time course for PYK2 activation in response to Ang II is consistent with the notion that PYK2 is upstream of ERK1 and ERK2 in the VSMC, since the peak activation of PYK2 occurs at 2 minutes; however, the present study and others have shown that maximal ERK1/2 activation by Ang II occurs at 5 minutes.

The results obtained from the present study suggest that Ca2+- and/or PKC-dependent pathways mediate Ang II-induced phosphorylation of PYK2. PYK2 was rapidly phosphorylated in response to the Ca2+ ionophore, ionomycin (Figure 7A). Moreover, chelation of cytosolic Ca2+ by pretreatment with BAPTA-AM resulted in a significant inhibition of Ang II–induced PYK2 phosphorylation. These data indicate that, like nonmuscle cells, one or more Ca2+-dependent steps are necessary for PYK2 activation in VSMC. This conclusion is supported by recent data from Brinson et al, who demonstrated that chelation of extracellular Ca2+ by EGTA partially blocked Ang II–induced PYK2 activation in VSMC. Because Ca2+ chelation only partially inhibited Ang II–induced PYK2 phosphorylation, our results suggest that other signaling pathways are involved. We provide evidence in the present study that activation of PKC is also sufficient to induce PYK2 phosphorylation, because PMA robustly stimulated PYK2 phosphorylation without having any appreciable effect on cytosolic Ca2+ (Figure 8).

To gain further insight into the requirement of PKC in Ang II signaling, we treated cells with PDBU to deplete PKC. PDBU pretreatment partially blocked PYK2 phosphorylation induced by Ang II (Figure 9B) suggesting the involvement of the phorbol ester-responsive, classical, and novel PKC isoforms (α, γ, ε, and δ). The incomplete inhibition of Ang II–induced PYK2 phosphorylation by PDBU pretreatment may indicate that the PDBU-insensitive PKC isoforms (β, λ, ι, and ζ) also play a role in mediating Ang II–induced tyrosine phosphorylation of PYK2. This possibility is supported by studies of Liao et al who demonstrated that MAP kinase regulation in VSMC by Ang II is partially mediated by the atypical PKC-ζ isoform. However, because the downregulation of PKC-α and PKC-ε by PDBU was not complete (~95%), we cannot exclude the possibility that the remaining amounts of these isoforms could also contribute to the PDBU-insensitive PYK2 activation in response to Ang II. Further studies are needed to determine the role of each PKC isoform in Ang II–induced PYK2 activation in VSMC.

Ang II has at least 2 important effects on VSMC. Ang II acts acutely as a potent vasoconstrictor and has longer term effects on gene expression that are believed to be involved in determining the hypertrophic phenotype. As noted above, the latter effects may involve PYK2 and the MAP kinase pathway. There is also a potential role for PYK2 in the acute vasoconstrictor actions of Ang II. PYK2 has been shown previously to induce phosphorylation and inhibition of delayed rectifier K+ channels. Clément-Chomienne et al have recently reported that Ang II treatment leads to inhibition of delayed rectifier K+ currents in rabbit portal vein myocytes. Inhibition of K+ currents is believed to lead to membrane depolarization and activation of voltage-sensitive Ca2+ channels, thereby contributing to the [Ca2+]i increase that activates the contractile response. Ang II may induce this acute response via activation of PYK2 and phosphorylation of K+ channels.

In summary, the present study characterizes the expression and activation of PYK2 in VSMC and suggests that PYK2 may represent the tyrosine kinase–linking, AT1-receptor–dependent increases in [Ca2+]i, and PKC activation to the MAP kinase pathway. Because Ang II is both a potent hypertrophic agonist and a vasoconstrictor in VSMC, understanding the role of PYK2 in Ang II signal transduction may provide insight into the cellular mechanisms that regulate VSMC growth and contractility.

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References


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