PYK2/PDZ-RhoGEF Links Ca\(^{2+}\) Signaling to RhoA

Zhekang Ying, Fernanda R.C. Giachini, Rita C. Tostes, R. Clinton Webb

Objective—Ras homolog gene family member A (RhoA)/Rho-kinase-mediated Ca\(^{2+}\) sensitization is a critical component of constrictor responses. The present study investigates how angiotensin II activates RhoA.

Methods and Results—Adenoviral vectors were used to manipulate the expression of regulator of G protein signaling (RGS) domain containing Rho-specific guanine exchange factors (RhoGEFs) and proline-rich tyrosine kinase 2 (PYK2), a nonreceptor tyrosine kinase, in primary rat vascular smooth muscle cells. As an evidence of RhoA activation, RhoA translocation and MYPT1 (the regulatory subunit of myosin light chain phosphatase) phosphorylation were analyzed by Western blot. Results showed that overexpression of PDZ-RhoGEF, but not p115-RhoGEF or leukemia-associated RhoGEF (LARG), enhanced RhoA activation by angiotensin II. Knockdown of PDZ-RhoGEF decreased RhoA activation by angiotensin II. PDZ-RhoGEF was phosphorylated and activated by PYK2 in vitro, and knockdown of PDZ-RhoGEF reduced RhoA activation by constitutively active PYK2, indicating that PDZ-RhoGEF links PYK2 to RhoA. Knockdown of PYK2 or PDZ-RhoGEF markedly decreased RhoA activation by A23187, a Ca\(^{2+}\) ionophore, demonstrating that PYK2/PDZ-RhoGEF couples RhoA activation to Ca\(^{2+}\).


Key Words: angiotensin II ■ RhoA ■ Ca\(^{2+}\) sensitization ■ PDZ-RhoGEF ■ PYK2
ously described,14 and maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C under a 95% air/5% CO2 atmosphere. VSMCs with no more than 5 passages were used in all experiments.

**Constructs**

Rat PDZ-RhoGEF, p115RhoGEF, and LARG cDNA were cloned from cultured rat aortic vascular smooth muscle cells by RT-PCR. Human PYK2 full-length cDNA was cloned from HEK293 cells by RT-PCR. These PCR products were inserted into pGEM-T. The inserts were verified by sequencing. To be easily measured, they were tagged with human influenza hemagglutinin (HA) at 5′, and their overexpression adenoviral vectors expressing were generated using Adeno-X Expression Systems 2 (Clotech). HA-tagged constitutively active PYK2 composed of the whole kinase domain was generated with a similar method. Myc-tagged human PDZ-RhoGEF was a gift of Dr Wedegaertner (Department of Microbiology and Immunology, Thomas Jefferson University). siRNA adenoviral vectors were generated using a system from Ambion (Philadephia, Penn.) according to the manufacturer’s instruction. PYK2 siRNA target sequence was described previously.15 PDZ-RhoGEF siRNA target sequence was: sense strand, 5′-CCCCAUCAU UCCUCACCA-3′; antisense strand, 5′-UGUGGAGGAAUG AUGGGG-3′.

**Transduction**

Rat VSMCs, COS-7, or NIH3T3-L1 cells in 60-mm dishes (around 90% confluent) were used to perform transduction. Briefly, cells were incubated with adenoviral vectors at a multiplicity of infection (MOI) of 40 PFU per cell for 4 hours, and then washed once with OptiMEM medium. Finally, 3 mL of culture medium was then added. After 40 to 48 hours, these cells were stimulated with angiotensin II or ionomycin, and cell lysates were then prepared.

**Western Blotting**

Cells were grown to confluence in 60-mm dishes, and then infected with adenoviral vectors. After 40 to 48 hours of infection, cells were treated with angiotensin II (100 nmol/L) or ionomycin (1 μmol/L), and then immediately placed on ice and washed twice with ice-cold PBS. All subsequent manipulations were performed on ice. To measure translocation, samples were homogenized as previously described.16 Otherwise, samples were lysed with 100 μL of ice-cold RIPA buffer (Upstate: 50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 50 mmol/L β-glycerophosphate, 50 mmol/L NaF, 1 mmol/L EGTA, 1 mmol/L Na3VO4, 1% NP-40, 0.25% sodium deoxycholate, 1 mmol/L PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 2 μg/mL pepstatin). Cells were scraped with a rubber policeman, rocked for 30 minutes at 4°C, and centrifuged at 14 000g for 30 minutes at 4°C. The supernatants were then used for Western blotting with standard techniques. The primary antibodies used in the present study were as follows: monoclonoal anti–β-actin (Sigma), rabbit anti-PYK2 (Sigma), rabbit anti–PDZ-RhoGEF (Alpha Diagnostics), monoclonoal antiphosphotyrosine (Upstate), monoclonoal antimmuc (Roche Applied Sciences), rat anti-human influenza hemagglutinin (anti-HA, Roche Applied Sciences), monoclonoal anti-RhoA (BD Biosciences), rabbit anti-phosphoMYPT1 (Santa Cruz), monoclonoal anti-MYPT1 (BD Biosciences).

**In Vitro Phosphorylation**

Myc-tagged PDZ-RhoGEF was overexpressed in HEK293T cells, purified by affinity chromatography (Roche Applied Science), and phosphorylated at 37°C in a total of 50 μL buffer containing 200 ng myc-tagged PDZ-RhoGEF, 1 U constitutively active PYK2 (Cycllex), 60 mmol/L HEPES-NaOH (pH 7.5), 3 mmol/L MgCl2, 3 mmol/L MnCl2, 3 μmol/L Na-orthovanadate, 1.2 mmol/L DTT, 10 μmol/L ATP. Reaction was stopped by adding 50 μL staurosporine (20 μmol/L).

**GEF Activity Assay**

GEF activity of PDZ-RhoGEF was measured with RhoGOF exchange assay Biochem Kit (Cytoskeleton) according to the manufacturer’s instruction.

RhoA activation assay: GTP bound RhoA in cell lines were measured with G-LISA Small G protein Activation Assays Kit (Cytoskeleton) according to the manufacturer’s instruction.

**Statistical Analysis**

The results are shown as mean±SEM, and n represents the number of animals per cell cultures used in the experiments. Statistical analyses were performed using 1-way ANOVA or Student t test. Post hoc comparisons were performed using Bonferroni test. Western blot data were analyzed by 1-sample t test, and the probability value was computed from the t ratio and the numbers of degrees of freedom. Values of P<0.05 were considered statistically significant.

**Results**

PDZ-RhoGEF (PRG), LARG, and p115RhoGEF were overexpressed in primary rat VSMCs with respective adenoviral vectors to determine their contribution to RhoA activation, on stimulation with angiotensin II. Figure 1A through 1C show that the overexpression of PDZ-RhoGEF, but not LARG or p115RhoGEF, significantly increased angiotensin II–induced RhoA translocation and MYPT1 phosphorylation. Notably, immunofluorescence analysis revealed that angiotensin II transiently induced translocation, from the cytosol to the membrane, of overexpressed PDZ-RhoGEF, but not the closest homolog, LARG (please see the supplemental materials, available online at http://atvb.ahajournals.org). This result was then confirmed by western blot analysis (Figure 1D and 1E).

Because the overexpression study indicated that PDZ-RhoGEF mediated angiotensin II–induced RhoA activation, PDZ-RhoGEF siRNA adenoviral vector was then generated to investigate the role of endogenous PDZ-RhoGEF in angiotensin II–induced RhoA activation. The PDZ-RhoGEF siRNA vector reduced PDZ-RhoGEF protein expression by more than 90% in rat VSMCs (Figure 2A and 2B), and markedly decreased angiotensin II–induced RhoA translocation and MYPT1 phosphorylation (Figure 2A, 2C, and 2D), supporting that PDZ-RhoGEF is necessary in angiotensin II–induced RhoA activation. To verify the specificity of this PDZ-RhoGEF siRNA vector, a human PDZ-RhoGEF expression adenoviral vector was generated, which had distinct sequences at the target site (please see the supplemental materials). Coinfection of human PDZ-RhoGEF overexpression vector and rat PDZ-RhoGEF siRNA vector revealed that the overexpression of human PDZ-RhoGEF was not affected by rat PDZ-RhoGEF siRNA and markedly restored angiotensin II–induced RhoA translocation and MYPT1 phosphorylation, clearly demonstrating the specificity of this PDZ-RhoGEF siRNA (Figure 2E through 2G).

Tyrosine phosphorylation is involved in the activation of many RhoGEFs.17 To test whether tyrosine phosphorylation is involved in angiotensin II–induced RhoA activation, PDZ-RhoGEF was immunoprecipitated from angiotensin II–treated rat VSMCs. Figure 3A shows that angiotensin II markedly induced tyrosine phosphorylation of PDZ-RhoGEF. Notably, angiotensin II also evidently increased tyrosine...
phosphorylation of another protein with a molecular weight of around 130 Kd. A previous study showed that PDZ-RhoGEF was precipitated by anti-PYK2.13 After membrane stripping and revisualization with anti-PYK2, results demonstrated that this protein was PYK2 (Figure 3A). The coimmunoprecipitation of PDZ-RhoGEF and PYK2 was further confirmed by coexpression of myc-tagged PDZ-RhoGEF and HA-tagged PYK2 in HEK293T cells (please see the supplemental materials).

To determine whether PYK2 regulates PDZ-RhoGEF activity through tyrosine phosphorylation, PDZ-RhoGEF was immunoprecipitated from rat VSMCs and then phosphorylated by constitutively active PYK2 in vitro. Figure 3B demonstrates that treatment with PYK2 markedly increased the tyrosine phosphorylation of PDZ-RhoGEF, in conjunction with a marked increase in the GEF activity. The essential role of tyrosine phosphorylation in PDZ-RhoGEF activation by PYK2 was further supported by results showing that without ATP, PYK2 did not increase either tyrosine-phosphorylation or GEF activity of PDZ-RhoGEF (please see the supplemental materials).

To examine the role of PYK2 in angiotensin II–induced RhoA activation, we also generated PYK2 siRNA adenoviral vector. Figure 3C through 3D shows that the PYK2 siRNA vector reduced PYK2 protein expression by more than 75% (Figure 3C and 3D), and markedly decreased angiotensin II–induced RhoA translocation and MYPT1 phosphorylation (Figure 3C, 3E, and 3F), supporting that PYK2 is also necessary for angiotensin II–induced RhoA activation.

To evaluate whether PYK2 activates RhoA via phosphorylation of PDZ-RhoGEF in living cells, constitutively active PYK2 expression adenoviral vector was also generated. The overexpression of this constitutively active PYK2 in rat VSMCs markedly induced RhoA translocation and MYPT1 phosphorylation, and these effects were markedly reduced by PDZ-RhoGEF siRNA (Figure 3G). Taken together, these results strongly indicate that on stimulation with angiotensin II, PYK2 phosphorylates PDZ-RhoGEF, resulting in RhoA activation in living cells.

Previous studies demonstrated that Ca²⁺ signaling is necessary and even sufficient to activate PYK2.18 Therefore, we examined whether angiotensin II activated RhoA in a Ca²⁺-dependent manner. Results show that membrane-permeant Ca²⁺ chelator, AM-BAPTA completely abolished angiotensin II–induced RhoA activation and MYPT1 phosphorylation (please see the supplemental materials), indicating that RhoA activation by angiotensin II is also Ca²⁺-dependent. To test whether Ca²⁺ activates RhoA through PYK2/PDZ-RhoGEF pathway, we analyzed the effect of PYK2 or PDZ-RhoGEF siRNA on RhoA activation on stimulation with ionomycin, a
Ca\(^{2+}\) ionophore. Figure 4A through 4C show that knockdown of either PYK2 or PDZ-RhoGEF markedly decreased ionomycin-induced RhoA translocation and MYPT1 phosphorylation, supporting that Ca\(^{2+}\)/H\(_{11001}\) activates RhoA through PYK2/PDZ-RhoGEF pathway. Because Ca\(^{2+}\) and RhoA are ubiquitous intracellular signal transducers, we finally tested whether PYK2/PDZ-RhoGEF linked Ca\(^{2+}\) signaling to RhoA in other cells. The expression levels of PYK2 and PDZ-RhoGEF in 6 different cell lines were examined by Western blot analysis. Figure 4D demonstrates that except for NIH-3T3-L1 (low PYK2 and PDZ-RhoGEF) and COS-7 cells (low PYK2), PYK2 and PDZ-RhoGEF were expressed at comparable levels in other cells (HEK293T, MDCK, Neuron2A, and PC12). Accordingly, ionomycin activated RhoA in test cells, except for NIH-3T3-L1 and COS-7 (Figure 4D). Expression of PDZ-RhoGEF in COS-7 cells or coexpression of PYK2 and PDZ-RhoGEF in NIH-3T3-L1 cells markedly restored RhoA activation by ionomycin(Figure 4E), further supporting that PYK2 and PDZ-RhoGEF are necessary to link Ca\(^{2+}\) to RhoA activation.

**Discussion**

Angiotensin II is one of the most potent vasoconstrictors, and its action is partially mediated by RhoA/Rho-kinase–induced Ca\(^{2+}\) sensitization.\(^{19–21}\) In the present study, we have identified specific signaling components that mediate angiotensin II–induced RhoA activation. Our results indicate that (1) PDZ-RhoGEF is necessary for RhoA activation by angiotensin II; (2) RhoA activation by angiotensin II also requires PYK2; (3) PYK2 activates RhoA through phosphorylation of PDZ-RhoGEF; (4) PYK2/PDZ-RhoGEF couples Ca\(^{2+}\) signaling to RhoA.

Because RGS domain can serve as a molecular bridge between heteromeric G proteins (primarily G12/13) and RhoGEF,\(^7\) RGS domain containing RhoGEFs, PDZ-RhoGEF, p115RhoGEF, and LARG are believed to play a role in RhoA activation on agonist binding to G protein–coupled receptors (GPCRs). Our present data indicate PDZ-RhoGEF, but not p115RhoGEF and LARG, mediates angiotensin II–induced RhoA activation, as evidenced by the different effects on angiotensin II–induced RhoA activation when they were knocked-down or overexpressed. Moreover, treatment with angiotensin II induced translocation of PDZ-RhoGEF, but not LARG. Because cellular membrane is the primary location for RhoA activation,\(^{22}\) this result strongly supports that PDZ-RhoGEF plays a significant role in angiotensin II–induced RhoA activation.

Consistent with the role of PDZ-RhoGEF in angiotensin II–induced RhoA activation, our present data indicate that protein–protein interaction through RGS domain may not be
involved in angiotensin II–induced RhoA activation. First, tyrosine phosphorylation is sufficient to activate PDZ-RhoGEF in vitro, suggesting that RGS domain-mediated protein–protein interaction is not necessary. Second, RhoA activation by angiotensin II is abolished by AM-BAPTA, indicating that it is Ca\(^{2+}\)-dependent. Although there is no evidence that RGS domain-mediated protein–protein interaction requires Ca\(^{2+}\), studies have shown that activation of PYK2 is Ca\(^{2+}\)-dependent.\(^1\) Third, PYK2 siRNA markedly reduced RhoA activation by angiotensin II, indicating a crucial role of PYK2 in angiotensin II–induced RhoA activation. Our results are also consistent with studies showing that although coupled to the same G\(\alpha\) subunit,\(^2\) GPCRs use distinct RGS domain-containing RhoGEFs.\(^3,4\) Taken together, these data indicate that mechanisms other than RGS domain-mediated protein–protein interaction is important in regulating RGS domain-containing RhoGEFs.

The present study shows that tyrosine phosphorylation by PYK2 is sufficient to activate PDZ-RhoGEF. Although tyrosine phosphorylation has been shown to regulate RhoGEFs, such as VAVs,\(^5\) this is, to our knowledge, the first study showing that PDZ-RhoGEF activity is regulated by tyrosine phosphorylation. In line with these results, angiotensin II–induced PDZ-RhoGEF tyrosine phosphorylation in VSMCs,\(^1\) and genistein, a nonspecific tyrosine kinase inhibitor, almost abolished angiotensin II–induced RhoA translocation in rat VSMCs (data not shown). Although the mechanism remains unclear, tyrosine kinase inhibitors have been shown to attenuate constrictive responses to vasoconstrictors.\(^6,7\) Therefore, our results may also provide a molecular basis for these investigations.

PYK2 is regulated by various extracellular signals that activate GPCRs or elevated cytoplasmic Ca\(^{2+}\), such as angiotensin II.\(^8,9\) PYK2 deficiency markedly reduces RhoA activation by chemokines in macrophages, suggesting that PYK2 is involved in RhoA activation.\(^10\) The present study shows that knockdown of PYK2 markedly reduced angiotensin II–induced RhoA activation. This is consistent with previous studies showing that knockdown of PYK2 by antisense oligonucleotides abolished various angiotensin II–
induced cellular responses.\textsuperscript{30,32} The essential role of PYK2 in angiotensin II–induced RhoA activation is also supported by the observation that PYK2 activates PDZ-RhoGEF through tyrosine phosphorylation in vitro. Further, a complex containing both PYK2 and PDZ-RhoGEF is suggested by the coimmunoprecipitation. These results together indicate that subsequent to PYK2 activation on stimulation with angiotensin II, PDZ-RhoGEF is phosphorylated by PYK2 and in turn activates RhoA. However, we cannot exclude that other kinases are also involved in this signaling, in particular Src which can work as an amplifier during PYK2 activation.\textsuperscript{33}

Consistent with that Ca\textsuperscript{2+} signaling is sufficient to activate RhoA in VSMCs,\textsuperscript{34} our results show that Ca\textsuperscript{2+} chelator abolished angiotensin II–induced RhoA activation. Moreover, knockdown of PYK2 or PDZ-RhoGEF markedly reduced RhoA activation by Ca\textsuperscript{2+} ionophore, indicating that PYK2/PDZ-RhoGEF couples Ca\textsuperscript{2+} signaling to RhoA. Because of the essential role of Ca\textsuperscript{2+} in vasoconstriction, these data indicate that PYK2/PDZ-RhoGEF may mediate RhoA activation on stimulation with various constrictors. However, because cultured rat VSMCs did not respond to many tested constrictors, it remains to be verified with other techniques, such as gene targeting.

In summary, we have identified PYK2 and PDZ-RhoGEF as the specific signaling components that mediate RhoA activation by angiotensin II. PYK2/PDZ-RhoGEF is sufficient to link Ca\textsuperscript{2+} signaling to RhoA, thereby providing a potential mechanism for constrictor responses.

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Disclosures
None.

References


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