Divergent kinase signaling mediates agonist-induced phosphorylation of phosphatase inhibitory proteins PHI-1 and CPI-17 in vascular smooth muscle cells

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Submitted 26 July 2005; accepted in final form 27 October 2005

Pang, Huan, Zhenheng Guo, Zhongwen Xie, Wen Su, and Ming C. Gong. Divergent kinase signaling mediates agonist-induced phosphorylation of phosphatase inhibitory proteins PHI-1 and CPI-17 in vascular smooth muscle cells. Am J Physiol Cell Physiol 290: C892–C899, 2006.—Phosphatase holoenzyme inhibitor (PHI)-1 is one of the newest phosphatase holoenzyme inhibitor and is highly expressed in smooth muscle cells.

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REVERSIBLE PHOSPHORYLATION of the myosin regulatory light chain, the primary mechanism regulating smooth muscle contraction, is coordinately regulated by myosin light chain kinase and myosin phosphatase (12). Although it is established that Ca\(^{2+}\)-calmodulin is primarily responsible for the activation of myosin light chain kinase, early studies assumed that myosin phosphatase activity was unregulated. However, more recent studies have clearly demonstrated that myosin phosphatase is inhibited on excitatory agonist stimulation (18, 27). Such agonist-induced inhibition of myosin phosphatase activity increases the sensitivity of the myosin phosphorylation toward Ca\(^{2+}\), thereby allowing force development under constant Ca\(^{2+}\). This is often referred to as “Ca\(^{2+}\) sensitization.” Ca\(^{2+}\) sensitization plays an important physiological role in agonist-induced smooth muscle contraction (27). Abnormalities in Ca\(^{2+}\) sensitization have been implicated in the pathophysiology of several cardiovascular disorders, including hypertension, coronary artery spasms, and arterial restenosis (15, 20, 26, 32). However, the mechanisms that couple excitatory agonist stimulation to the inhibition of myosin phosphatase are not yet fully understood. At least three mechanisms have been proposed: 1) direct binding and inhibition by CPI-17 (PKC-potentiating phosphatase inhibitor of 17 kDa), 2) phosphorylation of regulatory myosin phosphatase by rho kinase (ROCK), and 3) dissociation of phosphatase subunits. The recent identification of phosphatase holoenzyme inhibitor (PHI)-1 provides an additional possible mechanism leading to the inhibition of myosin phosphatase (5).

PHI-1 is an ortholog of CPI-17 (5) and is preferentially expressed in smooth muscle and endothelium in adult organisms (31). PHI-1 plays a potentially important role in regulating smooth muscle contraction (2) and endothelial and epithelial cell migration and retraction (30). Phosphorylation of PHI-1 at Thr57 increases protein phosphatase-1 (PP1) inhibitor potency (5). In purified enzyme systems, several kinases, including PKC, integrin-linked kinase (ILK), and ROCK can phosphorylate PHI-1 (2, 5). Interestingly, a very recent study (3) showed that angiotensin II and guanosine 5′-O-(3-thiotriphosphate) (GTP\(^\gamma\)S) induce PHI-1 phosphorylation in chicken smooth muscle tissues. However, it is unknown whether PHI-1 becomes phosphorylated in response to agonists other than angiotensin II and which kinase(s) couples agonist stimulation to PHI-1 phosphorylation.

CPI-17 is another phosphorylation-dependent myosin holoenzyme inhibitor and is highly expressed in smooth muscle (8). Accumulating evidence suggests that CPI-17 plays an important role in myosin light chain phosphatase inhibition and Ca\(^{2+}\) sensitization regulation (8, 27, 35). Interestingly, PKC, ROCK, and ILK, in addition to their ability to phosphorylate PHI-1, have also been reported to phosphorylate CPI-17 in isolated enzyme systems. Similar smooth muscle distribution and myosin phosphatase holoenzyme inhibitory function suggest a possible redundant role for PHI-1 and CPI-17. However, a recent study by Tountas et al. (31) found that PHI-1 and CPI-17 have distinct subcellular localizations, indicating that
PHI-1 and CPI-17 may be differentially regulated in intact smooth muscle cells. Accordingly, the present study tested whether divergent signaling pathways, in response to agonist stimuli, do indeed regulate PHI-1 and CPI-17 in vascular smooth muscle cells (VSMCs).

Our results show that agonists of G protein-coupled receptors (U-46619, angiotensin II, and thrombin) induce PHI-1 phosphorylation in primary cultured rat aortic VSMCs. In addition, divergent kinase signaling mediates U-46619 (thromboxane A2 receptor agonist)-induced PHI-1 and CPI-17 phosphorylation.

MATERIALS AND METHODS

Materials. Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN) and fed a standard diet and reverse osmosis water ad libitum until they were used. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. The cDNA of V14RhoA were kindly provided by Dr. Alan Hall (University College London, London, UK). The antibodies for total CPI-17 and Thr57 phosphorylated PHI-1 were kindly provided by Dr. Masumi Eto (University of Virginia, Charlottesville, VA). The antibodies for PKC-α and PKC-δ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DMEM was purchased from Invitrogen (Carlsbad, CA). PMA and bisindolylmaleine I hydrochloride (GF-109203X) were purchased from Sigma (St. Louis, MO). U-46619, Y-27632, H-1152, and thrombin were purchased from Calbiochem (San Diego, CA). ReadyStrip IPG Strips were purchased from Bio-Rad (Hercules, CA), and calf intestinal alkaline phosphatase (CIP) was acquired from New England Biolabs (Beverly, MA). Collagenases I and II were obtained from Worthington Biochemical (Lakevood, NJ). Other chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Primary cell culture. Rat aortic VSMCs were isolated by enzymatic dissociation as described previously (23, 24). Briefly, thoracic aortas were removed and washed in Hanks’ balanced salt solution containing 100 U/ml penicillin and 100 μg/ml streptomycin. After being cut into three pieces, the vessels were digested in 2 mg/ml collagenase I and then in 2 mg/ml collagenase II plus 0.5 mg/ml elastase for 30 min each at 37°C. The isolated cells were cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air-5% CO2. The first five passages of cells were used for the present studies because expressions of contractile signaling proteins, including PHI-1, CPI-17, rhoA, and ROCK, are not significantly affected in the initial five passages (24).

Two-dimensional PAGE. Primary rat aortic smooth muscle cells were starved in serum-free DMEM for 24 h and then treated with various inhibitors and/or agonist as indicated. After stimulation, the cells were fixed with cold 10% TCA and frozen rapidly in liquid nitrogen to preserve protein phosphorylation. TCA was then removed by washing the cells three times with cold acetone; the cells were then homogenized in SDS sample buffer, and proteins were quantified by bicinchoninic acid protein assay (Pierce). A total of 180 μg of protein from each sample were treated with a ReadyPrep 2-D cleanup kit (Bio-Rad) to remove salts, detergent, lipids, and phenolic compounds. The samples were then reconstituted in rehydration/sample buffer (Bio-Rad) and loaded onto a 7-cm IPG strip [isoelectric point (pI) 3–6, immobilized pH gradient] for rehydration overnight. The loaded IPG strips were transferred to the Protean IEF focusing tray, and isoelectric focusing electrophoresis was carried out with a Protean IEF Cell (Bio-Rad) according to the manufacturer’s instructions. After isoelectric focusing was completed, the IPG strips were equilibrated in equilibration buffer (Bio-Rad) and loaded into SDS-PAGE gels, and then two-dimensional (2D) gel electrophoresis was run. Once the position of the PHI-1 spots was determined in the preliminary experiments, only a portion of the IPG strip with the PHI-1 spots was grafted and multiple portion IPG strips were loaded onto SDS-PAGE gels to determine PHI-1 phosphorylation levels in subsequent experiments.

Immunoblot analysis. Proteins from primary cultured rat aortic smooth muscle cells were denatured using TCA. The same amount of proteins was separated by 2D-PAGE and SDS-PAGE. Specific proteins were detected by immunoblotting as described previously (11). Special care was taken to ensure that the immunoblot signals were within linear range. The signals were captured with a Kodak Image Station 4000 MM and quantified with Kodak Molecular Imaging software. PHI-1 phosphorylation levels were quantified using the ratios of phosphorylated PHI-1 (the spot with pI of 4.67) vs. total PHI-1 (sum of phosphorylated and unphosphorylated PHI-1). CPI-17 phosphorylation levels were quantified using the ratios of phosphorylated vs. total CPI-17 signals from two sets of parallel immunoblots.

RESULTS

Agnostins induce PHI-1 phosphorylation. To determine whether agonists induce PHI-1 phosphorylation in smooth muscle cells, we first tested whether 2D gel electrophoresis can separate phosphorylated and unphosphorylated PHI-1. With an antibody that recognizes total PHI-1, including phosphorylated and unphosphorylated PHI-1, two spots were detected at the reported apparent PHI-1 molecular mass in resting vascular smooth muscle cells (Fig. 1A, top; Ref. 5). By quantifying the ratio of the densities of the two PHI-1 spots, we found that ~91% of PHI-1 showed a more alkaline pI, consistent with the theoretical pI of unphosphorylated PHI-1 of 4.74, and ~9% of PHI-1 showed a more acidic pI, consistent with the theoretical pI of monophosphorylated PHI-1 of 4.67. This suggests that a 2D gel system can separate phosphorylated and unphosphorylated PHI-1. To further test whether PHI-1 indeed can be phosphorylated in intact smooth muscle cells and whether phosphorylation shifts its pI, we treated the cells with a membrane-permeant phosphatase inhibitor, calyculin A. We found that the more acidic PHI-1 spot increased from 9% to 91% (n = 3) with calyculin A (100 nM 30 min) treatment.
This suggests that the more acidic spot is phosphorylated PHI-1, that PHI-1 is in an active phosphorylation-dephosphorylation cycle in resting smooth muscle cells, and that blocking dephosphorylation is sufficient to drive nearly all PHI-1 to the phosphorylated state. Interestingly, on U-46619 stimulation, the more acidic PHI-1 was increased (Fig. 1A, bottom), indicating that U-46619 increases PHI-1 phosphorylation. U-46619-induced PHI-1 phosphorylation became detectable at 1 min, reached the highest level at 5 min, and remained at the highest level at 10 min. Therefore, a 10-min time point was chosen in the subsequent experiments. To further confirm that the more acidic spot represents the phosphorylated PHI-1, we determined 1) whether phosphatase treatment abolishes this acidic spot and 2) whether an antibody raised against Thr57 phosphorylated PHI-1 selectively recognizes this acidic spot. As shown in Fig. 1B, bottom, the acidic spot could not be detected after alkaline phosphatase treatment, leaving only the more alkaline spot. Moreover, the antibody selective for Thr57 phosphorylated PHI-1 recognized only the acidic spot at pI of 4.67 (Fig. 1C, bottom). Collectively, these data indicate that the two PHI-1 spots in 2D gels indeed correspond to the phosphorylated and unphosphorylated forms of PHI-1 and that U-46619 stimulation can increase PHI-1 phosphorylation in VSMCs.

To determine whether agonists in addition to U-46619 induce PHI-1 phosphorylation, VSMCs were stimulated with angiotensin II or thrombin. As shown in Fig. 2, similar to U-46619, angiotensin II and thrombin induced a significant increase in PHI-1 phosphorylation, although they were less potent. Compared with only 9% of PHI-1 phosphorylation under resting conditions, stimulation by angiotensin II (100 nM, 30 min), thrombin (1 U/ml, 20 min), or U-46619 (1 μM, 10 min) increased PHI-1 phosphorylation up to 18%, 18%, and 30%, respectively.
Minimal role of ROCK in U-46619-induced PHI-1 phosphorylation. Because ROCK has been shown to phosphorylate PHI-1 in an isolated enzyme system (2) and the RhoA/ROCK pathway is activated with U-46619, angiotensin II, or thrombin stimulation (23, 25), we tested whether ROCK is the kinase mediating agonist-induced PHI-1 phosphorylation. We blocked ROCK activity with two structurally distinct ROCK inhibitors, Y-27632 and H-1152, to determine whether ROCK is required for U-46619-induced PHI-1 phosphorylation. In addition, we expressed constitutively active mutant V14RhoA to activate ROCK and determine whether ROCK activation is sufficient to induce PHI-1 phosphorylation.

To ensure that ROCK mediates U-46619-induced CPI-17 phosphorylation in rat aortic VSMCs as we recently reported in rabbit aortic smooth muscle cells (23), we first tested whether ROCK phosphorylation in rat aortic VSMCs as we recently reported in rabbit aortic smooth muscle cells (23), we first tested whether ROCK phosphorylation is required for U-46619-induced PHI-1 phosphorylation.

We then tested whether, under the same conditions, Y-27632 or H-1152 inhibits U-46619-induced PHI-1 phosphorylation. Neither Y-27632 nor H-1152 significantly inhibited U-46619-induced PHI-1 phosphorylation (Fig. 3, A and B), preincubation of rat aortic VSMCs with 10 μM Y-27632 or 0.3 μM H-1152 for 30 min significantly inhibited U-46619-induced CPI-17 phosphorylation. We then tested whether, under the same conditions, Y-27632 or H-1152 inhibits U-46619-induced PHI-1 phosphorylation. Neither Y-27632 nor H-1152 significantly inhibited U-46619-induced PHI-1 phosphorylation (Fig. 3, C and D), indicating that ROCK is not responsible for U-46619-induced PHI-1 phosphorylation. To further test whether activation of endogenous ROCK phosphorylates PHI-1, we expressed a constitutively active mutant, V14RhoA, using an inducible adenoviral expression system (23). As shown in Fig. 4, A and B, no significant PHI-1 phosphorylation was observed on V14RhoA expression. In contrast, significant CPI-17 phosphorylation was observed on V14RhoA expression (Fig. 4, C and D), just as we had observed in rabbit aortic smooth muscle cells (23). This indicates that ROCK was indeed activated by V14RhoA in our system but did not phosphorylate PHI-1. These results, taken together, suggest that in rat aortic VSMCs, activation of endogenous ROCK is neither sufficient nor required for U-46619-induced PHI-1 phosphorylation.

PKC plays a critical role in U-46619-induced PHI-1 phosphorylation. Because PKC has been shown to phosphorylate PHI-1 in isolated enzyme systems, we then investigated the role of PKC in U-46619-induced PHI-1 phosphorylation. We inhibited PKC with a pan-PKC inhibitor, GF-109203X or by downregulation of PKC protein expression with long-term PMA treatment to determine whether PKC is required for U-46619-induced PHI-1 phosphorylation. In addition, we activated PKC by acute PMA treatment to determine whether PKC activation is sufficient to induce PHI-1 phosphorylation.

To determine the effect of GF-109203X on U-46619-induced PHI-1 phosphorylation, the cells were incubated with 3 μM GF-109203X for 30 min and then stimulated with 1 μM U-46619 for 10 min. As shown in Fig. 5, A and B, GF-109203X abolished U-46619-induced PHI-1 phosphorylation. In contrast, the same GF109203X treatment had no significant effect on U-46619-induced CPI-17 phosphorylation (Fig. 5, C and D). To inhibit PKC by downregulation, the cells were first treated with PMA (1 μM) for 48 h and then PKC protein expression and U-46619-induced PHI-1 phosphorylation were determined. As shown in Fig. 6A, consistent with previous reports (14, 33), the protein expression levels of PKC-α and PKC-β, the two isoforms of PKC examined, were significantly decreased by the 48-h PMA treatment. Importantly, in cells in which PKCs were downregulated, U-46619 failed to induce significant PHI-1 phosphorylation (Fig. 6, B and C). To the contrary, U-46619-induced CPI-17 phosphorylation was not affected by downregulation of PKCs (Fig. 6, D and E), which is consistent with our recent report (23) that PKC plays a minimal role in agonist-induced CPI-17 phosphorylation in rabbit aortic VSMCs. To further test whether direct PKC activation induces PHI-1 phosphorylation, we acutely stimulated the cells with PMA (1 μM, 5 min). Our results show that direct PKC activation by PMA is sufficient to induce significant PHI-1 phosphorylation to 2.4 ± 0.21-fold (n = 3) of the control level, which is similar to the phosphorylation level observed when cells were stimulated with angiotensin II or

![Minimal role of ROCK in U-46619-induced PHI-1 phosphorylation](image)

![PKC plays a critical role in U-46619-induced PHI-1 phosphorylation](image)

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**A** CPI-17 phosphorylation (fold increase) in response to U-46619 (1 μM), Y-27632 (10 μM), H-1152 (0.3 μM), or vehicle (DMSO). **B** Summary of the immunoblots in A showing the inhibitory effect of Y-27632 or H-1152 on U-46619-induced CPI-17 phosphorylation. **C** Representative immunoblots of 3 independent experiments are shown. **D** Summary of the immunoblots in C showing the lack of effect of Y-27632 or H-1152 on U-46619-induced PHI-1 phosphorylation. Representative immunoblots of 4 independent experiments are shown.

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thrombin. Varying PMA concentrations (1 μM or 100 nM) or PMA stimulation times (5, 15, or 30 min) were unable to increase PHI-1 phosphorylation any further (data not shown). Together, these data suggest that PKC plays a critical role in U-46619-induced PHI-1 phosphorylation.

To test the possibility that some agonist selectively activates PHI-1 or CPI-17 phosphorylation, we determined whether angiotensin II and thrombin, two agonists shown to phosphor-
ylate PHI-1 (Fig. 1), induce CPI-17 phosphorylation in the same preparations. The results show that angiotensin II and thrombin, similarly to U-46619, induced phosphorylation of both PHI-1 and CPI-17.

**DISCUSSION**

The major novel findings of the current study are that 1) we have demonstrated for the first time that PHI-1 is phosphorylated in response to U-46619, angiotensin II, and thrombin stimulation in primary cultured rat aortic smooth muscle cells and 2) we have identified for the first time that divergent kinase signaling mediates U-46619-induced phosphorylation of PHI-1 and CPI-17. PKC is primarily responsible for PHI-1 phosphorylation, whereas ROCK is primarily responsible for CPI-17 phosphorylation.

**PHI-1 phosphorylation in response to agonist stimulation.** It was recognized that activation of phosphorylation-dependent PP1 by G protein-coupled receptor agonist stimuli is one of the major mechanisms by which agonist inhibits myosin phosphatase and thereby induces smooth muscle contraction (28). PHI-1 and CPI-17 are the two most recently identified members of the superfamily of PP1 inhibitory proteins (5, 8), with phosphorylation required to increase their protein phosphatase inhibitory potency. Although CPI-17 has been subjected to
intensive study (1, 4, 6, 7, 9, 10, 16, 17, 19, 21–23, 29, 34), little is known about the function and regulation of PHI-1. In an isolated enzyme system (2, 5), it was well documented that PHI-1 can be phosphorylated by several kinases, including PKC, ROCK, and ILK (2, 5). However, in vivo, such as in primary cultured VSMCs, it was largely unknown whether agonist could induce PHI-1 phosphorylation and, if so, which kinase was responsible for agonist-induced PHI-1 phosphorylation. Our data provide the first convincing evidence that in primary cultured VSMCs, PHI-1 is phosphorylated by G protein-coupled receptor agonists angiotensin II, U-46619, and thrombin and that PKC is the kinase that mediates U-46619-induced PHI-1 phosphorylation.

In isolated enzyme systems, PHI-1 can be doubly phosphorylated at two sites by PKC and ROCK: Thr57 and an unidentified serine (2, 5). However, only phosphorylation at Thr57, a site that is preferentially phosphorylated, has been shown to enhance the phosphatase inhibitory potency of PHI-1. Thus, in rat aortic VSMCs, it is important to determine which site is phosphorylated in response to agonist stimulation. Two independent results from the present study suggest that Thr57 is the likely site phosphorylated in PHI-1 in response to agonist stimulation. First, only a single phosphorylation spot, in addition to an unphosphorylated spot, was detected using a PHI-1 antibody that recognizes phosphorylated and unphosphorylated PHI-1 with 2D-PAGE (Figs. 1 and 2). Second, this phosphorylated spot was recognized by an antibody raised against Thr57 phosphorylated PHI-1. Together, our data show that PHI-1 is phosphorylated at Thr57 in response to excitatory agonist stimulation, suggesting that PHI-1 plays a potential role in agonist-induced inhibition of myosin phosphatase.

Divergent kinase signaling mediates PHI-1 and CPI-17 phosphorylation. Only 29% of protein sequences are identical between PHI-1 and CPI-17, but the amino acid sequences around the phosphorylation site Thr57 in PHI-1 and Thr38 in CPI-17 are highly conserved and nearly identical (5). Thus a kinase that can phosphorylate one is theoretically expected to have the ability to phosphorylate the other. Consistent with this concept, it has been demonstrated that PKC, ROCK, and ILK can phosphorylate PHI-1 as well as CPI-17 in isolated enzyme systems where the kinases have free access to their substrates (2, 5). However, our results clearly show that U-46619-induced PHI-1 and CPI-17 phosphorylation are mediated by divergent kinase signaling in intact smooth muscle cells. For example, inhibition of ROCK by Y-27632 or H-1152 selectively diminished U-46619-induced CPI-17 phosphorylation without significantly affecting U-46619-induced PHI-1 phosphorylation (Fig. 3). In contrast, inhibition of PKC activity by GF-109203X (Fig. 5) or by PKC downregulation (Fig. 6) selectively diminished U-46619-induced PHI-1 phosphorylation without significantly affecting U-46619-induced PHI-1 phosphorylation (Fig. 3). How is this selectivity achieved in smooth muscle cells? One possible mechanism is by compartmentalization of the kinases and their substrates. Indeed, in smooth muscle cells, the localizations of PHI-1 and CPI-17 differ significantly (31).

We note that El-Touhky et al. (3) reported very recently that phosphorylation of PHI-1 in chicken smooth muscle was suppressed by treatments with Y-27632 and GF-109203X. The reason for the difference between their results and ours is currently unclear but may be due to the use of different stimuli (GTPyS vs. U-46619) or the difference between chicken smooth muscle and rat smooth muscle. Chicken smooth muscle lacks CPI-17 (19, 29), so the signal pathway regulating myosin phosphatase might be different. In addition, PHI-1 subcellular localization might be different in different tissues.
latory mechanisms of myosin phosphatase might be unique in each smooth muscle tissue of various species. In addition, the present study has demonstrated that PKC is involved in U-46619-induced PHI-1 phosphorylation, but it did not identify the specific PKC isoform(s) that mediates U-46619-induced PHI-1 phosphorylation. Conventional and/or novel PKCs are likely involved, because inhibition of conventional and novel PKC by GF-109203X or by PKC downregulation significantly inhibited U-46619-induced PHI-1 phosphorylation. However, future studies are required to identify the specific PKC isoforms that are critical in U-46619-induced PHI-1 phosphorylation.

Functional implications of agonist-induced PHI-1 and CPI-17 phosphorylation. Our data show that a single agonist, such as U-46619, thrombin, or angiotensin II, can induce phosphorylation of both PHI-1 and CPI-17. The reason why the cell uses two pathways to phosphorylate and activate similar functionally proteins is unclear at present. There are at least three possibilities. One possibility is that the two pathways act additively or synergistically to achieve maximal inhibition of myosin phosphatase and phosphorylation of myosin light chain. Indeed, both ROCK and PKC have been shown to mediate agonist-induced myosin light chain phosphorylation in smooth muscle (for review, see Refs. 13, 27). The second possibility is that PHI-1 and CPI-17 are located in different subcellular compartments and therefore may couple agonist stimulation to a distinct local increase in myosin phosphorylation, thereby providing the spatial coordination required for complex cellular functions such as cell migration. Indeed, PHI-1 and CPI-17 have been shown to have differential subcellular localizations (31). A third possibility is that PHI-1 and CPI-17 couple agonist stimulation to different cellular functions. For example, PHI-1 couples agonist stimulation to the regulation of glucose metabolism, whereas CPI-17 couples agonist stimulation to myosin light chain phosphorylation. Phosphorylated PHI-1 has been shown to inhibit glyco- gen-bound PP1 (8), whereas phosphorylated CPI-17 failed to do so (6). Future studies are required to test and distinguish these possibilities.

In summary, our data show for the first time that the G protein-coupled receptor agonists U-46619, angiotensin II, and thrombin induce PHI-1 phosphorylation in primary cultured rat aortic VSMCs. We demonstrate for the first time that U-46619 induces PHI-1 and CPI-17 phosphorylation through divergent kinase signaling: PKC is primarily responsible for PHI-1 phosphorylation, and ROCK is primarily responsible for CPI-17 phosphorylation. Our results therefore suggest that PHI-1 and CPI-17 may be involved in different cellular functions.

ACKNOWLEDGMENTS
We thank Dr. Masumi Eto for the generous gift of antibodies to total PHI-1 and to phospho-specific (Thr57) as well as for critical suggestions and comments on the manuscript. We thank the University of Kentucky Cardiovascular Research Group for invaluable advice and assistance.

GRANTS
This work was supported by National Heart, Lung, and Blood Institute Grant HL-67284 and a Career Development Award from the American Diabetes Association (to M. C. Gong) and a Scientist Development Grant from the American Heart Association (to Z. Guo).

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