

Angiotensin II-induced aortic ring constriction is mediated by phosphatidylinositol 3-kinase/L-type calcium channel signaling pathway

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Abbreviations: Akt/PKB, Akt/protein kinase B; AngII, angiotensin II; PI3K, phosphatidylinositol 3-kinase; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; RASMC, rat aortic smooth muscle cell; VSMC, vascular smooth muscle cell

Abstract

Angiotensin II (AngII) is a crucial hormone that affects vasoconstriction and exerts hypertrophic effects on vascular smooth muscle cells. Here, we showed that phosphatidylinositol 3-kinase-dependent calcium mobilization plays pivotal roles in AngII-induced vascular constriction. Stimulation of rat aortic vascular smooth muscle cell (RASMC)-embedded collagen gel with AngII rapidly induced contraction. AngII-induced collagen gel contraction was blocked by pretreatment with a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) whereas ERK inhibitor (PD98059) was not effective. AngII-induced collagen gel contraction was significantly blocked by extracellular calcium depletion by EGTA or by nifedipine which is an L-type calcium channel blocker. In addition, AngII-induced calcium mobilization was also blocked by nifedipine and EGTA, whereas intracellular calcium store-depletion by thapsigargin was not effective. Finally, pretreatment of rat aortic ring with LY294002 and nifedipine significantly reduced AngII-induced constriction. Given these results, we suggest that PI3K-dependent activation of L-type calcium channels might be involved in

AngII-induced vascular constriction.

Keywords: calcium; calcium channels, L-type; muscle contraction; muscle, smooth; 1-phosphatidylinositol 3-kinase

Introduction

The renin-angiotensin system (RAS) plays crucial roles in many physiological processes. Angiotensin II (AngII) is formed from a series of proteolytic cleavages of angiotensinogen to angiotensin I (AngI) by renin, and subsequent cleavage of AngI to AngII by angiotensin converting enzyme (ACE). Acute roles for AngII have been implicated in the regulation of plasma volume control, vasoconstriction, and blood pressure control, whereas chronic stimulation with AngII induces hyperplasia and hypertrophy of vascular smooth muscle cells (VSMCs) (Geisterfer *et al.*, 1988).

Among its pleiotropic effects, vasoconstriction-induced blood pressure control is the major function of AngII. The receptors for AngII include angiotensin type 1 receptor (AT₁R) and angiotensin type 2 receptor (AT₂R), which belong to the seven trans-membrane superfamily that is coupled with G proteins. Occupation of AT₁R by AngII results in intracellular calcium mobilization, which binds to calmodulin and activates myosin light chain kinase (MLCK). Activated MLCK eventually leads to the phosphorylation of the myosin light chain which causes it to interact with actin, causing contraction of VSMCs (Yan *et al.*, 2003). MLCK is negatively regulated by myosin light chain phosphatase (MLCP), which is inactivated by Rho kinase. Activation of the Rho kinase signaling pathway leads to sustained contraction of VSMCs (Uehata *et al.*, 1997). Therefore, intracellular calcium mobilization is a key mediator of VSMC contraction.

Binding of AngII to AT₁R has been reported to activate several G proteins including G_{αq/11}, G_{α12/13}, and G_{βγ} complexes. Activation of G proteins leads to the activation of downstream signaling molecules such as PLC, PLD, and PLA₂ (Ushio-Fukai *et al.*, 1999a). Among the PLC isoforms, PLC-β isoform is coupled with G proteins and generates inositol tris-phosphate (IP₃) from phosphatidylinositol 4,5-

bisphosphate (PIP₂) by hydrolytic cleavage (Rhee, 2001). IP₃ evokes calcium mobilization by stimulating IP₃ receptors on intracellular calcium storage sites such as the endoplasmic reticulum. Elevation of intracellular calcium concentrations eventually leads to VSMC contraction. In addition to the effect of AngII on calcium mobilization, AngII also causes membrane NAD(P)H oxidase to generate reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H₂O₂). For instance, AngII causes NAD(P)H oxidase subunits Nox1 and Nox4 to generate ROS (Lassegue *et al.*, 2001), although the molecular mechanism is still not clear. AngII-dependent production of ROS affects the activation of many signaling molecules including p38 MAPK, Akt, and ERK1/2 (Ushio-Fukai *et al.*, 1999b; Nishida *et al.*, 2005). Although p38 MAPK and ERK1/2 are activated by AngII stimulation, these kinases are implicated in VSMC differentiation, proliferation and migration (Sugden and Clerk 1997; Taniyama *et al.*, 2004).

AngII also cross-communicates with several tyrosine kinases, including receptor tyrosine kinases such as EGF-R, PDGF-R, insulin receptor (IR), and nonreceptor tyrosine kinases such as the c-Src family kinases, calcium-dependent proline-rich kinase 2 (Pyk2), focal adhesion kinase (FAK) and Janus kinase (JAK). The major function of AngII-dependent activation of nonreceptor tyrosine kinases seems to be the regulation of focal adhesion complex formation and actin bundling

(Taniyama *et al.*, 2003). AngII can trans-activate receptor tyrosine kinases independent of growth factors. For instance, stimulation of VSMC with AngII stimulates formation of Shc and Grb2 complex with PDGF-R (Linseman *et al.*, 1995). Once activated, receptor tyrosine kinases evoke the phosphatidylinositol 3-kinase (PI3K)/3-phosphoinositide-dependent kinase-1 (PDK-1)/Akt cascade, which leads to growth, metabolism, survival, and remodeling of VSMCs.

The major physiological function of AngII is VSMC contraction and maintaining vascular tone through the regulation of calcium mobilization. Although PLC- β -dependent intracellular calcium mobilization during AngII stimulation is important for VSMC contractions, recent evidence also supports the idea that AngII increases myocardial activity through the augmentation of inward calcium currents through L-type calcium channels (Baker *et al.*, 1992). Other evidence has supported the argument that G $\beta\gamma$ directly activates PI3K γ isoform to generate PIP₃ (Viard *et al.*, 1999), which eventually stimulates L-type calcium channels in vascular myocytes from portal vein (Le Blanc *et al.*, 2004). However, there is no direct evidence that PI3K-dependent calcium mobilization is important for vascular constriction upon AngII stimulation. Here we provide strong evidence that AngII-dependent rat aortic ring constriction is mediated by PI3K-mediated L-type calcium channel activation.

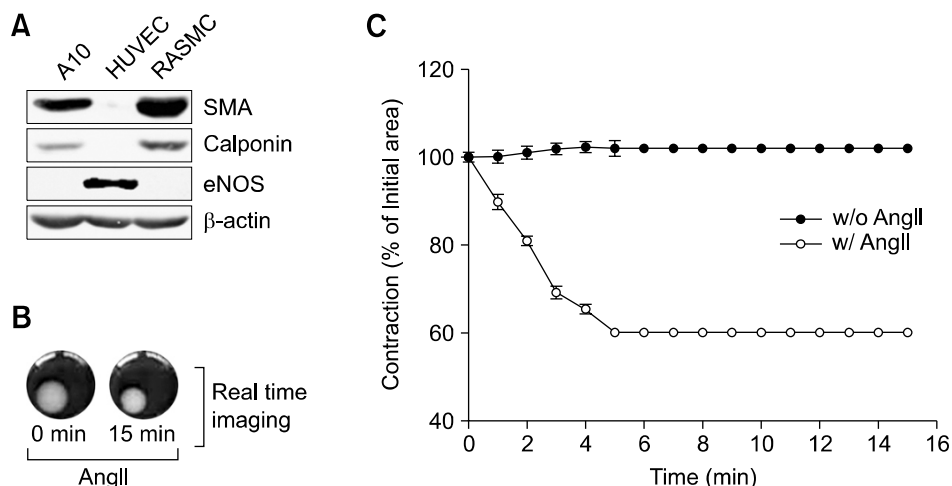


Figure 1. AngII-induced RASMC contraction. (A) Cells from rat aortic smooth muscle were subjected to western blot analysis using the indicated antibodies. A10 and HUVEC cell lysates were included as markers for smooth muscle and endothelial cells, respectively. (B) Isolated RASMCs were embedded in collagen gel matrix as described in Methods. Gel contraction was initiated by addition of AngII (1 μ M), and images were captured as described in Methods. Images are representative of two independent experiments each done in triplicate. (C) RASMC-embedded collagen gel was stimulated with either vehicle or AngII (1 μ M), images were taken, and the surface area of the collagen gel was analyzed as described in Methods. Data are mean \pm S.D. of two independent experiments ($n = 3$ for each experiment).

Results

AngII-induced contraction of isolated RASMCs

Previously, it was reported that AngII regulates calcium mobilization through PI3K- β -dependent L-type calcium channel activation in vascular myocytes from portal vein (Viard *et al.*, 1999). To understand the role of PI3K-dependent L-type calcium channel modulation in VSMCs from thoracic aorta, we isolated rat aortic smooth muscle cells (RASMCs). As shown in Figure 1A, isolated RASMC expressed vascular smooth muscle cell marker proteins such as smooth muscle actin (SMA) and calponin. On the other hand, RASMCs did not express endothelial nitric oxide synthase (eNOS) which is an endothelial cell marker protein. A10 and human umbilical vein endothelial cell (HUVEC) extracts were used as positive controls of smooth muscle and endothelial cells, respectively. Isolated RASMCs rapidly contracted within 5 min after exposure to 1 μ M of AngII (Figure 1B and 1C). However, AngII did not induce the contraction of A10 and HUVEC cells (data not shown).

PI3K- and L-type calcium channel-dependent RASMC contraction

Since PI3K is involved in the AngII-induced calcium mobilization of smooth muscle cells from portal vein (Viard *et al.*, 1999), we next examined the possible role of PI3K in VSMC contractions. As shown in Figure 2A, pretreatment with the PI3K

inhibitor, LY294002 (10 μ M), significantly reduced AngII-induced RASMC contractions. However, inhibition of the ERK1/2 signaling pathway by PD98059 (10 μ M) slightly potentiated AngII-induced RASMC contractions. Inhibition of PLC by U73122 (10 μ M) also blocked AngII-induced RASMC contraction (data not shown). Since calcium mobilization is important for smooth muscle cell contraction, we examined the effect of calcium channel blocker on AngII-induced RASMC contractions. As shown in Figure 2B, chelation of extracellular calcium by EGTA (5 mM) or blocking L-type calcium channels by nifedipine (20 μ M) strongly inhibited AngII-induced RASMC contraction. However, depletion of intracellular calcium by thapsigargin (1 μ M) did not affect AngII-induced RASMC contraction.

Inhibition of calcium mobilization by PI3K and L-type calcium channel inhibitors

Since PI3K and L-type calcium channel inhibitors strongly blocked AngII-induced RASMC contraction, we examined the effect of these inhibitors on intracellular calcium mobilization. As shown in Figure 3A, pretreatment of RASMC with LY294002 completely blocked AngII-induced calcium mobilization, whereas pretreatment of PD98059 did not affect AngII-induced calcium mobilization. AngII-induced calcium mobilization was also completely blocked by extracellular calcium chelation (Figure 3B). Inhibition of L-type calcium channels by nifedipine significantly attenuated AngII-induced calcium mobilization.

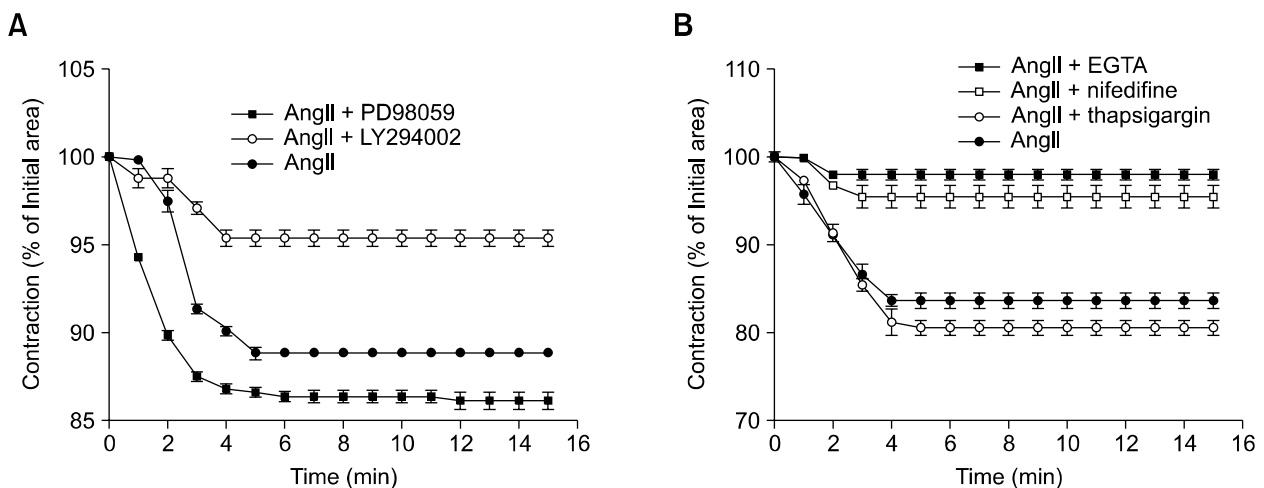


Figure 2. PI3K and L-type calcium channels are important for RASMC contraction *in vitro*. (A) RASMCs embedded in collagen gel matrix were pretreated with vehicle, LY294002 (10 μ M), or PD98059 (10 μ M) for 20 min, and then stimulated with AngII (1 μ M). Data are mean \pm S.D. of two independent experiments ($n = 3$ for each experiment). (B) RASMCs embedded in collagen gel matrix were pretreated with vehicle, EGTA (5 mM), thapsigargin (1 μ M), or nifedipine (20 μ M) for 20 min, and then stimulated with AngII (1 μ M). Data are mean \pm S.D. of two independent experiments ($n = 3$ for each experiment).

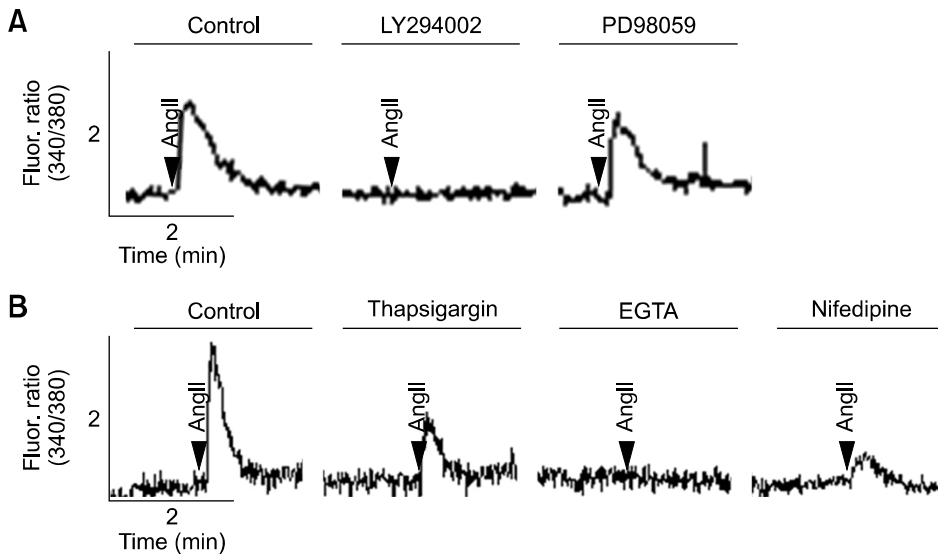


Figure 3. AngII induces calcium mobilization through PI3K- in an L-type calcium channel-dependent manner. (A) RAMSCs were pretreated with vehicle, LY294002 (10 μ M), or PD98059 (10 μ M) for 20 min, followed by stimulation with AngII (1 μ M). Images are representative of three independent experiments. The time of AngII stimulation is indicated by an arrow head. (B) RASMCs were pretreated with vehicle, thapsigargin (1 μ M), EGTA (5 mM), or nifedipine (20 μ M) for 20 min, followed by stimulation with AngII (1 μ M). Images are representative of three independent experiments. The time of AngII stimulation is indicated by an arrow head.

PI3K- and L-type calcium channel-dependent constriction of rat aorta

Since calcium mobilization and contraction of RASMC were mediated by PI3K and L-type calcium channels, we extended the experiments to *ex vivo* conditions to clarify the effect of PI3K and L-type calcium channels on vascular constriction. As shown in Figure 4A, stimulation of thoracic aorta with AngII resulted in about 30-35% contraction of vessels. AngII-induced vascular contraction was significantly blocked by a PI3K inhibitor, LY294004, whereas inhibition of ERK1/2 signaling pathway did not affect AngII-induced vascular

contractions. Depletion of extracellular calcium by EGTA strongly inhibited AngII-induced vascular contraction (Figure 4B). In addition, blocking of L-type calcium channels by nifedipine significantly inhibited AngII-induced vascular contractions. In contrast, depletion of intracellular calcium stores by thapsigargin did not affect AngII-induced vascular contraction.

Discussion

A plethora of evidence suggests that AngII plays

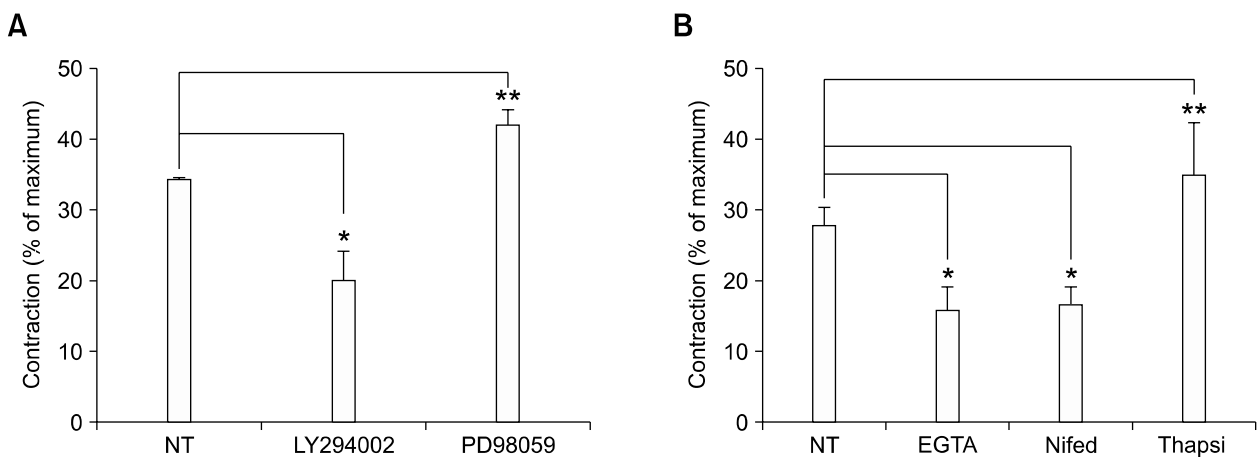


Figure 4. PI3K and L-type calcium channels regulate AngII-induced contractions of thoracic aorta. (A) Thoracic aortic rings were isolated from rats and pretreated with vehicle alone, LY294002 (10 μ M), or PD98059 (10 μ M) for 20 min, and then stimulated with AngII (1 μ M). Contraction is expressed as a percent of KCl (60 mM)-induced maximum tension. Data are mean \pm S.D. of two independent experiments ($n = 2$ for each experiment). *Significantly different from vehicle-pretreated aortic rings ($P < 0.05$). **Values were not statistically different from vehicle-pretreated aortic rings ($P > 0.05$). (B) The AngII (1 μ M)-induced tension of thoracic aortic rings was measured after pretreatment with vehicle, EGTA (5 mM), nifedipine (20 μ M), or thapsigargin (1 μ M) for 20 min.

key roles in the regulation of blood pressure. AngII acutely increases blood pressure by induction of blood vessel constriction. In fact, antagonists of AT₁R effectively reduced blood pressure in hypertensive rats (Dendorfer *et al.*, 2005). It has been reported that AngII activates many signaling pathways including the PLC, Ras/MEKK/MAPK, and p38 MAPK pathways (Smith *et al.*, 1984; Meloche *et al.*, 2000; Ishihata *et al.*, 2002). In addition, AngII stimulates PI3K through the modulation of G_{βγ} (Viard *et al.*, 1999). Likewise, pharmacological inhibition of PI3K resulted in the attenuation of RASM contraction as well as in calcium mobilization (Figure 2-4). However, MAPK signaling pathway seems not to be involved in AngII-dependent physiological responses. Currently, the involvement of MAPK signaling pathways in VSMC contractions is controversial. Nevertheless, our results clearly showed that pharmacological inhibition of MAPK signaling pathway did not affect VSMC contraction both *in vitro* and *ex vivo* (Figure 2A and 4A). More importantly, inhibition of MAPK signaling pathway did not alter AngII-induced calcium mobilization (Figure 3A), which is a very important signaling response during AngII-induced vascular smooth muscle contraction. Therefore, the MAPK signaling pathway seems to be involved in prolonged responses such as proliferation and differentiation (Marrero *et al.*, 1997; Yamada *et al.*, 1999) rather than in acute responses.

AngII-induced calcium mobilization is a crucial event during VSMC contraction. Apparently, AngII stimulates PLC isoforms to generate IP₃ through the activation of G_{αq/11}, G_{12/13}, and G_{βγ}. Calcium is released from endoplasmic reticulum via IP₃-operated calcium channels. Hence, PLC-mediated calcium mobilization has been implicated in the regulation of VSMC contraction. The possible involvement of PLC was suggested by experiments in which electroporation of anti-G_{αq/11} antibody to VSMC suppressed AngII-induced IP₃ production (Ushio-Fukai *et al.*, 1998). However, there is no direct link between IP₃-operated calcium mobilization and VSMC contraction. In contrast, our data showed that depletion of intracellular calcium stores by thapsigargin inhibited 50% of AngII-induced calcium mobilization (Figure 3B). This result indicates that AngII partially stimulates IP₃-operated calcium mobilization. However, blocking IP₃-operated calcium mobilization was not enough to block AngII-induced VSMC contractions both *in vitro* and *ex vivo* (Figure 2B and 4B). These results suggest that another mechanism of calcium mobilization is important for VSMC contractions. Currently, the involvement of PLC-mediated signaling pathways in VSMC contractions is still ambiguous. Studies

using a genetically modified mouse model of PLC will provide detailed molecular mechanism of PLC-dependent VSMC contraction.

Although AngII-induced PLC activation and subsequent calcium mobilization have been implicated as major signaling pathways, it has been reported that AngII also induces the activation of growth factor receptor signaling pathways. For instance, stimulation of VSMC with AngII activates the tyrosine kinase signaling pathway including MAPK (Watts *et al.*, 1998). However, pharmacological inhibition of MAPK does not affect VSMC contraction. Likewise, our results showed that pharmacological inhibition of the MAPK signaling pathway affected neither AngII-induced VSMC contraction nor calcium mobilization (Figure 2A, 3A and 4A). The involvement of other signaling pathways rather than G protein-coupled PLC activation has been implicated by recent studies. The atypical PI3K class is directly activated by the G_{βγ} subunits of G proteins (Viard *et al.*, 1999). Consistent with this, our results showed that pharmacological inhibition of PI3K strongly attenuated AngII-induced calcium mobilization and VSMC contraction (Figure 3A and 4A). PI3K-dependent activation of calcium mobilization was further demonstrated by Le Blanc *et al.* (2004). They showed that PIP₃, which is a product of PI3K, can directly activate L-type calcium channels. It is also supported by our results that depletion of extracellular calcium or blocking of L-type calcium channels significantly blocks AngII-induced calcium mobilization (Figure 3B). In addition, the link between L-type calcium channel opening and VSMC contraction was further confirmed by showing that inhibition of L-type calcium channels blunted AngII-induced VSMC contraction both *in vitro* and *ex vivo* (Figure 2B and 4B). Therefore, it is quite clear that direct activation of L-type calcium channels by PIP₃ is important for the regulation of VSMC contraction. The downstream effector molecule of PIP₃ seems not to be involved in AngII-dependent VSMC contractions since pharmacological inhibition of Akt/PKB by SH-5 (10 μM) did not affect AngII-induced VSMC contraction (data not shown).

Because inhibition of PLC activity by U73122 also suppressed AngII-induced VSMC contraction (data not shown) we cannot exclude possible involvement of PLC activation in AngII-dependent VSMC contraction. Consistent with this, depletion of intracellular calcium stores by thapsigargin resulted in a 50% reduction of AngII-induced calcium mobilization (Figure 3B), demonstrating PLC-mediated calcium mobilization in AngII-stimulated VSMCs. Nonetheless, pretreatment of thapsigargin barely affected AngII-induced VSMC contractions

(Figure 4B). These results could be interpreted as follows. First, it is possible that depletion of intracellular calcium stores is not sufficient for the dropping effective concentration of calcium to evoke VSMC contraction. Second, in addition to calcium mobilization, another signaling pathway can be diverted from PLC, and lead the VSMC contraction. Consistent with this idea, it has been reported that PKC also regulates AngII-induced VSMC contractions (Yu *et al.*, 2004). Therefore, multiple signaling pathways seem to be involved in the regulation of AngII-induced VSMC contractions.

In summary, we have demonstrated that AngII-induced aortic ring constriction is mediated by PI3K-dependent regulation of L-type calcium channel activity. Our studies suggest that changes in intracellular calcium concentration by L-type calcium channels are important for arterial smooth muscle contraction, which is important for *in vivo* responses in AngII-dependent acute blood pressure regulation. The analysis of calcium modulation using knock-out animal models will shed more light on the AngII-mediated signaling pathway of VSMC contraction.

Methods

Reagents

All cell culture media and supplements were purchased from Cambrex Corp. Anti-SMA and anti-calponin antibodies were purchased from Sigma-Aldrich. Anti- β -actin antibody was obtained from MP Biomedicals. Anti-eNOS antibody was from BD Transduction Laboratories. Collagen gel was purchased from BD Bioscience and Fura-2-AM was from Invitrogen. LY294002 and PD98059 were obtained from Calbiochem. Nifedipine, thapsigargin, collagen, gelatin, and AngII were purchased from Sigma-Aldrich. All other high quality reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Rat aortic smooth muscle cell (RASMC) preparation

Pathogen-free Sprague-Dawley rats (6 to 8 weeks old) were housed under diurnal lighting conditions and allowed unlimited access to feed and tap water. All animal experiments were performed under university animal welfare guidelines. Aortic smooth muscle cells were isolated from the thoracic aorta by a tissue explanting method as described previously (Hall *et al.*, 1991). Briefly, sodium barbital anesthetized rats were perfused with PBS for 5 min. Thoracic aorta was aseptically isolated and surrounding fat and connective tissues were discarded. Vessels were longitudinally cut and the lumen side was scraped with a razor blade to remove the intima. Vessels were fragmented into 3-5 mm lengths and explanted lumen side down on collagen-coated culture dishes. Tissue fragments were maintained in DMEM/10% FBS with changing of the medium every two days. After seven days of explanting,

tissue fragments were discarded and sprouted VSMCs were collected and used for experiments.

Collagen gel contraction assay

To measure RASMC contractility, RASMCs from confluent cultures were trypsinized, resuspended in serum free DMEM and diluted to 1×10^6 cells/ml. The cell suspension was mixed on ice with collagen gel solution (6 mg/ml of collagen type I in $2 \times$ PBS pH8.0) to give 5×10^5 cells/ml and 3 mg/ml of collagen gel solution. One hundred μ l of RASMC-collagen gel solution was added per well to 24-well plates. Where indicated, various inhibitors were added. The plate was incubated at 37°C to allow for gel polymerization. After 1 h, the gels were floated with serum-free DMEM and AngII was added to initiate contraction while images were captured using a digital charge-coupled device camera. Collagen gel contraction was measured as a decrease in gel area using Scion Image software (compliments of Scion Corporation, Frederick, MD, <http://www.scioncorp.com>). Relative gel area was obtained by dividing the area at each time point by the initial area of the gel.

Measurement of cytosolic calcium concentration

Intracellular calcium concentration was measured using fura-2/AM, a calcium-sensitive fluorescent dye, as described previously (Gryniewicz *et al.*, 1985). Briefly, a total of 2×10^6 RASMCs were incubated with 3 μ M fura-2/AM at 37°C in fresh serum-free DMEM medium with stirring for 50 min. A total of 2×10^5 cells were aliquoted for each assay into Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, 2.2 mM $CaCl_2$, and 0.2 mM EGTA). Fluorescence was measured at emission wavelength of 500 nm using an excitation wavelength of 340 nm and 380 nm.

Measurement of aortic ring tension

The thoracic aorta was rapidly removed and immersed in ice-cold modified Krebs' solution (118 mM of NaCl, 4.7 mM of KCl, 1.2 mM of $MgSO_4 \cdot 7H_2O$, 1.2 mM of KH_2PO_4 , 25 mM of $NaHCO_3$, 2.5 mM of $CaCl_2 \cdot 2H_2O$, 5.6 mM of glucose, pH 7.4). The aorta was dissected to remove fat and connective tissue and sliced into rings (2-3 mm in length). The aortic rings were suspended by two L-shaped stainless-steel wires inserted into the lumen followed by immersion in 10 ml organ chambers filled with Krebs' solution. The basal tension of the aortic ring was maintained at 2 g and aerated continuously with 95% O_2 /5% CO_2 . Changes in isometric tension were recorded using a force-displacement transducer (Grass FT 0.3, Quincy, MA) connected to a Power Lab system 400 (ML 118, PowerLab, AD Instruments, Medford, MA) and saved in a computer. The aortic rings were allowed to equilibrate for 90 min while changing the chamber solution every 15 min. After resting tension of each vascular specimen had stabilized, maximum tension of each aortic ring was measured after addition of 60 mM KCl. Constricted aortic rings were relaxed by washing with Krebs' solution for 2 h. Various inhibitors were added 20 min prior to AngII stimulation.

AngII-induced contraction was expressed as percent of 60 mM KCl-induced contraction.

Western blot analysis

VSMCs were lysed in lysis buffer containing 20 mM Tris-HCl, pH 7.3, 1 mM EGTA/EDTA, 1% Triton X-100, 1 mM Na₃VO₄, 10 µg/ml leupeptin and 10 µg/ml aprotinin. After centrifugation at 12,000 rpm for 5 min, 30 µg/ml of total protein was loaded onto a 10% polyacrylamide gel and, after electrophoresis, transferred to nitrocellulose membranes. Membranes were incubated with the indicated primary antibodies and IR Dye-conjugated secondary antibody. Protein bands were visualized by an infrared image analyzer (Li-COR Bioscience).

Statistical analysis

Results are expressed as the mean ± S.D. of two independent experiments ($n = 3$ for each experiment). When two groups were compared, an unpaired Student's *t*-test was used to assess differences. *P*-values less than 0.05 were considered significant.

Acknowledgements

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