Involvement of Rho and Rho-Associated Kinase in Sphincteric Smooth Muscle Contraction by Angiotensin II

SATISH RATTAN,[1] RAJINDER N. PURI, AND YA-PING FAN

Department of Medicine, Division of Gastroenterology and Hepatology, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107

The tonic smooth muscles of lower esophageal sphincter (LES) and internal anal sphincter (IAS) are subject to modulation by the neurohumoral agents. We report that angiotensin (Ang) II-induced contraction of rat IAS and LES smooth muscle cells (SMC) was inhibited by *Clostridium botulinum* C3 exozyme, HA 1077 and Y 27632, suggesting a role for Rho kinase and a Rho-associated kinase (ROK). Ang II-induced contraction of the SMC was also attenuated by genistein, antibodies to the $pp60^{c\text{-}src}$, p^{190} RhoGTPase-activating protein (p^{190} RhoGAP), carboxyl terminus of $G\alpha_{13}$, carboxyl terminus peptide, and ADP ribosylation factor (ARF) antibody. Ang II-induced increase in p^{190} RhoGAP tyrosine phosphorylation was attenuated by genistein. Furthermore, Ang II-induced increase in smooth muscle tone and phosphorylation of myosin light chain (MLC; 20 kDa; MLC_{20}-P) were attenuated by Y 27632 and genistein. The results suggest an important role for $G\alpha_{13}$ and $pp60^{c\text{-}src}$ in the intracellular events responsible for the activation of RhoA/ROK in Ang II-induced contraction of LES and IAS SMC. Exp Biol Med 228:972–981, 2003

Key words: smooth muscle; angiotensin II; Rho-associated kinase; tyrosine phosphorylation; $pp60^{c\text{-}src}$; $G\alpha_{13}$; p^{190} RhoGAP

The tonic smooth muscles of internal anal sphincter (IAS) and lower esophageal sphincter (LES) and IAS serve important functions to prevent gastrointestinal reflux and anorectal incontinence, respectively (1–5). Although a majority of tone in these tissues is by virtue of the myogenic properties of the smooth muscles, the basal tone is prone to excitatory modulation to a number of neurohumoral substances (6–8). Our recent studies suggest that among different species, rat is an ideal model for the investigations of angiotensin (Ang) II effects in the IAS and LES (9). Our studies further suggest that Ang II-induced contraction of these smooth muscles is mediated via the activation of AT_1 receptor and involves multiple pathways that include Ca^{2+} influx, Protein kinase C (PKC), $^{44/42}$mitogen-activated protein kinases (MAPKs), and Rho kinase (8–10). The exact sequence of intracellular events from AT_1 receptor activation to the Rho kinase activation in the contraction of these smooth muscles is not known.

Rho belongs to ras superfamily of monomeric or small G-proteins (11–14) and is known to exert diverse cellular functions. Rho cycles between a biologically inactive GDP-bound state and an active GTP-bound state (11). The low intrinsic GTPase activity of Rho is accelerated by the GTPase-activating proteins (GAPs) (11, 13). The rate of GDP/GTP exchange is regulated by the guanine nucleotide exchange factors (GEFs) (11, 13). Two serine/threonine kinases, p^{160} ROCK and ROKα, containing similar kinase and coiled-coil structure domains, have been identified among different effectors of Rho (15). Rho- and Rho-associated kinase (ROK)-mediated signaling pathways play important roles in smooth muscle contraction (15–20). ROK inactivates myosin light chain (20 kDa; MLC_{20}) phosphatase by catalyzing phosphorylation of the p^{130} myosin-binding subunit in a Ca^{2+}-independent manner (21). ROK also phosphorylates MLC in a Ca^{2+}-independent manner at Ser-19, the site of MLC_{20} phosphorylation (MLC-P) by the Ca^{2+}-calmodulin-dependent MLC kinase (MLCK) (22). These two biochemical events may increase MLC-P at constant intracellular ($[Ca^{2+}]_i$), a phenomenon known as Ca^{2+} sensitization of MLC-P (22). G-protein-mediated Ca^{2+} sensitization of rabbit trachea smooth muscle has been shown to involve ROK (23). In addition to Rho, another monomeric G-protein ADP ribosylation factor (ARF) has also been shown to participate in signal transduction (19, 20, 24, 25).

The members of $G\alpha_{12}$ family ($G\alpha_{12/13}$) of GTPases appear not to affect any of the second messenger-generating

This work was supported by the National Institutes of Diabetes and Digestive and Kidney Diseases Grant DK-35385 and by an institutional grant from Thomas Jefferson University.

[1] To whom requests for reprints should be addressed at Department of Medicine, Division of Gastroenterology and Hepatology, Jefferson Medical College, Thomas Jefferson University, 1025 Walnut Street, Room 901 College, Philadelphia, PA 19107. E-mail: Satish.Rattan@mail.tju.edu

Received August 5, 2002.
Accepted April 11, 2003.

1535-3702/03/2288-0972$15.00

functions of $G\alpha_s$ and $G\alpha_i$ (26). Lysophosphatidic acid-induced growth stimulation but not stress fiber formation is inhibited by pertussis toxin (27). This suggests that stress fiber formation (via Rho activation) is mediated by a G-protein(s) different from G_i such as $G\alpha_{12}/G\alpha_{13}$ (28). The role of $G\alpha_{12}/G\alpha_{13}$ in different cell systems has been reviewed elsewhere (29, 30). $G\alpha_{12}/G\alpha_{13}$ have been shown to interact directly with p^{115} RhoGEF (31, 32) and to activate Rho.

There is only limited information examining the role of G-protein-coupled receptor (GPCR)-mediated signaling events in the activation of Rho and ROK, and ARF in gastrointestinal smooth muscle contraction (19, 20, 33, 34). Ang II has been shown to be a potent contractile agonist in different sphincteric smooth muscles, especially in the IAS (7–9, 35) in different species examined, including humans (36). In all of the species examined, Ang II has been shown to cause an increase in the basal LES tone in the concentration range found in the blood (37). Additionally, Ang II-converting enzyme activity has been shown to be present in the iris sphincter (38). Ang II is known to produce its actions, including smooth muscle contraction of the LES and IAS, via multiple signal transduction pathways (8, 9, 39). The purpose of the present investigation is to examine the role of Rho kinase pathway and related sequence of events in Ang II-induced contraction of these tonic smooth muscles. Such information is important for increased understanding of the basic mechanisms for the contraction of these smooth muscles.

Materials and Methods

Preparation of Smooth Muscle Strips and Measurement of Isometric Tension. Smooth muscle strips (1 × 10 mm) from LES and IAS of rats were prepared as described previously (40) in oxygenated (95% O_2 and 5% CO_2) Krebs' solution (118.07 m*M* NaCl, 4.69 m*M* KCl, 2.52 m*M* $CaCl_2$, 1.16 m*M* $MgSO_4$, 1.01 m*M* NaH_2PO_4, 25 m*M* $NaHCO_3$, and 11.10 m*M* glucose). The mucosal and submucosal layers were removed by sharp dissection. The LES and IAS circular smooth muscle strips thus prepared were used for the recording of isometric tension, using force transducer (model FT03; Grass Instruments, Quincy, MA) connected to a PowerLab recorder (CB Sciences, Milford, MA). The details of the procedure have been given before (40). The smooth muscle strips initially stretched with 10 mN (millinewton) force were allowed to equilibrate for 1 hr. During equilibration, the smooth muscle strips were replenished every 20 min with fresh solution. Only those smooth muscle strips that developed spontaneous steady tone and relaxed in response to electrical field stimulation were used in further experiments. The optimal length and the basal tone of the smooth muscle strips were determined as described previously. The increase in contraction in rat LES and IAS smooth muscle was calculated as percentage of maximal contraction induced by bethanechol (1×10^{-4} *M*) at the conclusion of the experiment, in relation to active tone, determined at the end of each experiment using 5 m*M* EGTA.

Use of animals was approved by the institution's Animal Care and Use Committee.

Isolation and Permeabilization of Smooth Muscle Cells (SMC) from Rat LES and IAS. SMC from the tonic smooth muscle strips were isolated by the method described previously (41). The sphincteric regions were identified and marked *in situ* using a water-perfused catheter assembly specially designed for rats and mice. The areas were marked as high-pressure zones that relaxed to appropriate stimuli. Rat LES and IAS smooth muscle strips were cut into small pieces (1- to 2-mm cubes) and were incubated in oxygenated Krebs' solution containing collagenase (0.01% for LES and 0.013% for IAS) and soybean trypsin inhibitor (0.01%) at 37°C for two successive 1-hr periods. After each incubation, the mixture was filtered through a 500-μm Nitex mesh. The tissue trapped on the mesh was rinsed with 25 ml (5 × 5 ml) of collagenase-free Krebs' solution. The tissue was finally incubated in collagenase-free Krebs' solution at 37°C, and dispersion of the cells (0-1 hr) was monitored periodically by examining a 10-μl aliquot of the mixture under microscope. SMC were harvested by filtration through the Nitex mesh. The filtrate containing the cells was centrifuged at 350*g* for 10 min at room temperature. The cells in the pellet were resuspended in Krebs' solution at a cell density of 3×10^4 cells/ml. To increase the cell yield, the LES and IAS tissue samples were pooled from three to four animals for different experiments. Each experimental protocol with an appropriate control was carried out under identical conditions.

Permeabilization of SMC was accomplished by the method previously used in our laboratory (40, 42). LES and IAS SMC were permeabilized by incubating them in cytosolic solution (20 m*M* NaCl, 100 m*M* KCl, 5 m*M* $MgSO_4$, 0.96 m*M* NaH_2PO_4, 25 m*M* $NaHCO_3$, 1 m*M* EGTA, 0.48 m*M* $CaCl_2$, and 1% bovine serum albumin [BSA]) with saponin (75 μg/ml) for 3 min at room temperature. The cell suspension was centrifuged at 350*g* for 10 min. The pellet was suspended in a cytosolic solution supplemented with antimycin A (10 m*M*), ATP (1.5 m*M*), phosphocreatine (5 m*M*), and creatine phosphokinase (10 U/ml) and was centrifuged at 350*g* for 10 min. Cells were washed twice with the modified cytosolic solution to remove saponin and were resuspended in the fresh modified cytosolic solution.

Measurement of SMC Length by Scanning Micrometry. Aliquots (30 μl) of the SMC were incubated with Ang II (5-min exposure) in the absence or presence of selective inhibitors (20 min of incubation before Ang II). Incubations were terminated by the addition of acrolein (0.1%). The mean length of 30 cells chosen randomly in each set was determined by micrometry using phase contrast microscopy. The images were stored digitally and the cell length was measured by the Image-Pro Plus V4.0 program (Media Cybernatics, Silver Spring, MD). Digital data was transferred directly to the Microsoft Excel computer

program. The data were expressed as the percentage of shortening of the original SMC length of 30 randomly chosen cells in each set as mean ± SEM. Each n in such experiments represents the mean of 30 cells either from one animal or cells pooled from more than one animal. Different categories of experiments were repeated at least four times in cells from different groups of animals ($n = 4$).

Gel Electrophoresis and Western Blot Analysis. While monitoring the isometric tension, the rat LES and IAS smooth muscles strips were quick-frozen either in the basal state, after Ang II (1×10^{-7} M) alone, or in the presence of selective inhibitors, at the time of maximal contraction (usually within 2 to 3 min of addition of Ang II). The inhibitors were added 10 to 20 min before Ang II challenge. The smooth muscle tissues were fixed at the time of sustained contraction with Ang II, before and after different inhibitors. Protein extracts from the smooth muscles were prepared by cutting the tissues into small pieces and incubating them with a homogenization buffer (1% SDS, 1 m*M* sodium orthovanadate, and 10 m*M* Tris, pH 7.4) at 90°C for 3 min. The incubation mixtures were homogenized followed by centrifugation at 16,000*g* for 15 min at 4°C. Protein contents in the supernatants were estimated by Lowry's method. Solutions of the protein extracts were prepared by mixing them with an equal volume of a 2× sample buffer (125 m*M* Tris, pH 6.8, 10% glycerol, 2% β-mercaptoethanol, and 0.006% bromphenol blue) and heating the samples in a boiling water bath for 3 min. Protein samples (40 μg of protein/20 μl) were subjected to SDS-PAGE by the method of Laemmli (43). Discontinuous gel system using 4% stacking gel, pH 6.8, and 10% running gel, pH 8.8, was used.

Proteins in the gels were electroblotted on to a nitrocellulose membrane at 100 V for 1 hr at 4°C. The membranes were transferred in a blocking buffer containing Tris-buffered saline (TBS; 20 m*M* Tris and 137 m*M* NaCl, pH 7.4), 1% BSA, and 0.1% Tween 20 and were left overnight at 4°C. The membranes were rinsed with a washing buffer containing TBS and 0.1% Tween 20 (TBS-Tween), treated with a primary antibody for 1 hr with gentle agitation, and washed three times with TBS-Tween. They were subsequently treated with a secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hr with gentle agitation and then washed three times with TBS-Tween.

The dilutions of primary and secondary antibodies were as follows: p^{190} RhoGAP (primary antibody, 1:1000) and an anti-mouse immunoglobulin (Ig) G-HRP (secondary antibody, 1:1000). Western blots of tyrosine-phosphorylated p^{190} RhoGAP were prepared by using mouse monoclonal anti-phosphotyrosine antibody (primary antibody, 1:1000) and an anti-mouse IgG-HRP (secondary antibody, 1:1000). MLC_{20} mouse monoclonal antibody (IgM class) to MLC_{20} (primary antibody, 1:500) and an anti-mouse IgM (μ-chain specific)-HRP (secondary antibody, 1:1000). Western blots of phospho-MLC_{20} were prepared by using mouse monoclonal antibody to antiphosphoserine (primary antibody, 1:8000) and an anti-mouse IgG-HRP (secondary antibody, 1:2000).

The membranes were blotted semi-dry by placing them in between two filter papers and they were developed with the enhanced chemiluminescence (ECL) Western blotting reagents according to the instructions provided by the supplier (Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands were visualized by exposing the membranes to x-ray films that were scanned with a scanner (model SNAPSCAN 310; Agfa, Ridgefield Park, NJ) followed by densitometric analysis by using the Image-Pro Plus V4.0 software (Media Cybernetics).

Data Analysis. Data were calculated as mean ± SEM using the Sigma Plot computer program for PCs. Differences between groups were examined by students t test (P value) with $P < 0.05$ considered significant. One-tail P values were computed by the same computer program.

Chemicals and Drugs. Ang II (human), HA 1077 (Rho kinase inhibitor) (44) and $G_{\alpha 13\text{-}CT}$ peptide (a blocking peptide corresponding to the carboxyl terminus of $G_{\alpha 13}$ protein) were obtained from Calbiochem (San Diego, CA). Tyrosine kinase inhibitor genistein and MEK inhibitor PD 98059 were obtained from Research Biochemicals International (Natick, MA) and Biomol (Plymouth Meeting, PA), respectively. Rho kinase inhibitor Y 27632 (18, 44) was a generous gift from Yoshitomi Pharmaceutical Industries (Osaka, Japan). *Clostridium botulinum* C3 3exozyme (known to specifically inactivate Rho by ADP-ribosylation; collagenase [CLS II] 140 U/mg) (45, 46), soybean trypsin inhibitor, ATP, antimycin A, creatine, creatine phosphate, creatine phosphokinase, bethanechol (carbamyl β-methyl choline chloride), and saponin were purchased from Sigma (St. Louis, MO). All other chemicals used in this investigation were of reagent grade. All materials used in electrophoresis experiments, including the molecular mass markers (broad range), were obtained from Bio-Rad Laboratories (Hercules, CA).

Antibodies. Anti- p^{190} RhoGAP (antibody to full length), and antiphosphotyrosine monoclonal antibodies, and anti-mouse antibody conjugated with HRP were obtained from Transduction Laboratories (Lexington, KY). Anti-$pp60^{c\text{-}src}$ polyclonal antibody was obtained from Chemicon International (Temecula, CA). Antiphosphoserine, antimyosin light chain (20 kDa; IgM class; MLC_{20}) monoclonal antibodies and anti-mouse (IgM, μ-chain)-HRP, and anti-sheep antibody-HRP were obtained from Sigma. $G\alpha_{13\text{-}CT}$ antibody (antibody to the carboxyl terminus of the $G\alpha_{13}$ subunit) was obtained from Calbiochem (St. Louis, CA). ARF antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to p^{115} RhoGEF was kindly provided by Dr. G. Bollag (ONYX, Richmond, CA). Anti-rabbit antibody-HRP, ECL western blotting reagent kit, and x-ray Hyper film were purchased from Amersham Pharmacia Biotech. Different agents and antibodies were used in the concentrations known to selectively block their respective actions (18, 44–49).

Results

Effect of ROK Inhibitors on Ang II-Induced Contraction of SMC from LES and IAS. Rho kinase inhibitors HA 1077 (1×10^{-6} *M*) and Y 27632 (1×10^{-6} *M*) alone caused no significant shortening of the SMC from LES and IAS ($P > 0.05$; $n = 4$; Fig. 1). Ang II (1×10^{-7} *M*) produced 18.5% ± 1.2% shortening of SMC from LES and 16.4% ± 1.5% those of the IAS. HA 1077 and Y 27632 inhibited Ang II-induced contraction of LES SMC to 2.6% ± 0.3% and 3.8% ± 0.4%, respectively, and 4.5% ± 0.4% and 3.2% ± 0.4%, respectively in the IAS ($*P > 0.05$; $n = 4$). These inhibitors in these concentrations caused no significant effect on PKC activity in the LES and IAS SMC (data not shown).

Effect of *C. botulinum* C3 Exozyme on Ang II-Induced Contraction of SMC from LES and IAS. SMC were permeabilized to facilitate the transport of large molecular mass molecules such as C3 exozyme, $pp60^{c\text{-}src}$, and p^{190} RhoGAP antibodies across the plasma membrane.

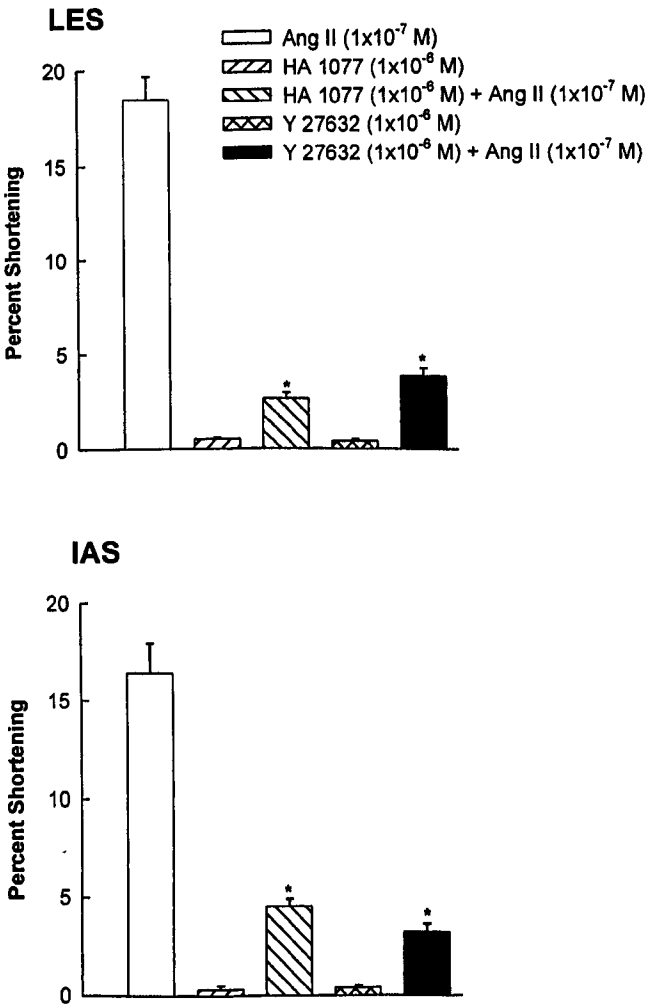

Figure 1. Effect of ROK inhibitors HA 1077 and Y 27632 on Ang II-induced contraction of SMC from LES and IAS. Pretreatment with HA 1077 and Y 27632 cause a significant attenuation ($*P < 0.05$; $n = 4$) of Ang II-induced shortening of the SMC. On the other hand, the inhibitors by themselves had no significant effect of the basal cell lengths by themselves.

Permeabilization of LES and IAS SMC had no significant effect on the SMC contraction induced by Ang II, bethanechol, and KCl (data not shown). The C3 exozyme (50 μg/ml) by itself caused no significant effect on the length of the permeabilized SMC from either the LES or IAS ($P > 0.05$; Fig. 2). Ang II-induced shortening of the permeabilized SMC from both LES (19.4% ± 1.5%) and IAS (18.6% ± 1.7%) was significantly ($*P < 0.05$; $n = 4$) inhibited by the C3 exozyme in SMC from these tissues (4.8% ± 0.5% and 5.7% ± 0.6%, respectively). Above data suggest a role for RhoA/ROK in Ang II-induced contraction of SMC from LES and IAS.

Effect of Antibodies to $pp60^{c\text{-}src}$ and p^{190} RhoGAP on Ang II-Induced Contraction of SMC from LES and IAS. Western blots obtained by using a polyclonal antibody to $pp60^{c\text{-}src}$ and monoclonal antibody to p^{190} RhoGAP showed the presence of these proteins in LES and IAS smooth muscles in their basal states (data not shown). Antibodies to $pp60^{c\text{-}src}$ and p^{190} RhoGAP (introduced into the cells by permeabilization) alone had no significant effect on the length of SMC from LES and IAS. In such experiments, Ang II-induced shortening of SMC from LES (21.3% ± 1.2%) and IAS (18.6% ± 1.4%; Fig. 3) was

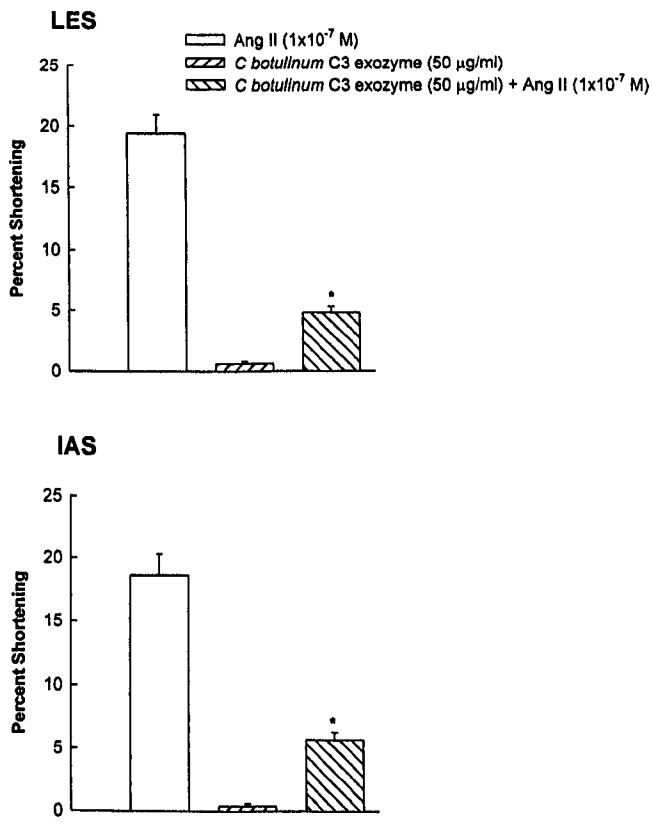

Figure 2. Effect of Rho inhibitor *Clostridium botulinum* C3 exozyme on Ang II-induced contraction of SMC from LES and IAS. Permeabilized SMC from LES and IAS were incubated with Ang II in the presence or absence of the C3 exozyme (50 μg/ml, 0.5 hr). Note that the C3 exozyme causes significant inhibition ($*P < 0.05$; $n = 4$) of Ang II-induced contraction of the SMC. However, by itself it has no significant effect on the basal cell lengths.

significantly inhibited by the antibodies to pp60$^{c\text{-}src}$ and p^{190} RhoGAP (4.6% ± 0.4% and 6.4% ± 0.8%, respectively; *$P < 0.05$; $n = 4$). For these studies, control experiments with Ang II were performed in the presence of nonimmune IgG (5 μg/ml). Herbimycin A (Hb A; c-src inhibitor; 1 × 10^{-6} M) produced inhibition of Ang II-induced contraction of the SMC similar to that by pp60$^{c\text{-}src}$ antibody. In the IAS, Ang II-induced shortening of SMC of 18.1% ± 2.1% was attenuated by Hb A to 6.5% ± 1.1% (*$P < 0.05$; $n = 4$).

Effect of PD 98059 and Genistein on Ang II-Induced Tyrosine Phosphorylation of p^{190} RhoGAP. Western blots prepared by using monoclonal antibody to phosphotyrosine show the presence of basal levels of tyrosine phosphorylated p^{190} RhoGAP in unstimulated rat LES and IAS (Fig. 4A). In addition, Ang II treatment caused an increase in the levels of tyrosine phosphor-

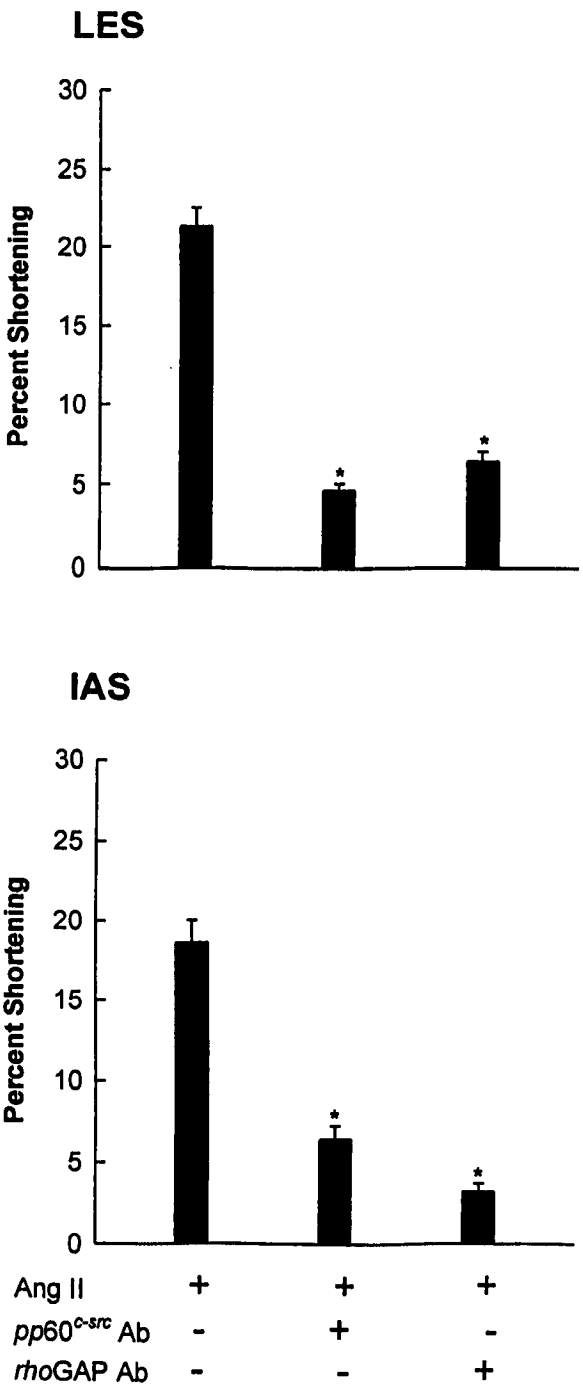

Figure 3. Effect of antibodies to pp60$^{c\text{-}src}$ and p^{190} RhoGAP on Ang II-induced contraction of SMC from LES and IAS. Control experiments with Ang II alone were carried out in the presence of nonimmune IgG (5 μg/ml). Permeabilized cells from LES and IAS were incubated with an antibody to p^{190} RhoGAP (5 μg/ml) for 1 hr at room temperature followed by Ang II for 5 min. Note that pp60$^{c\text{-}src}$ and p^{190} RhoGAP antibodies (5 μg/ml) cause significant attenuation in Ang II-induced contraction of SMC (*$P < 0.05$; $n = 4$).

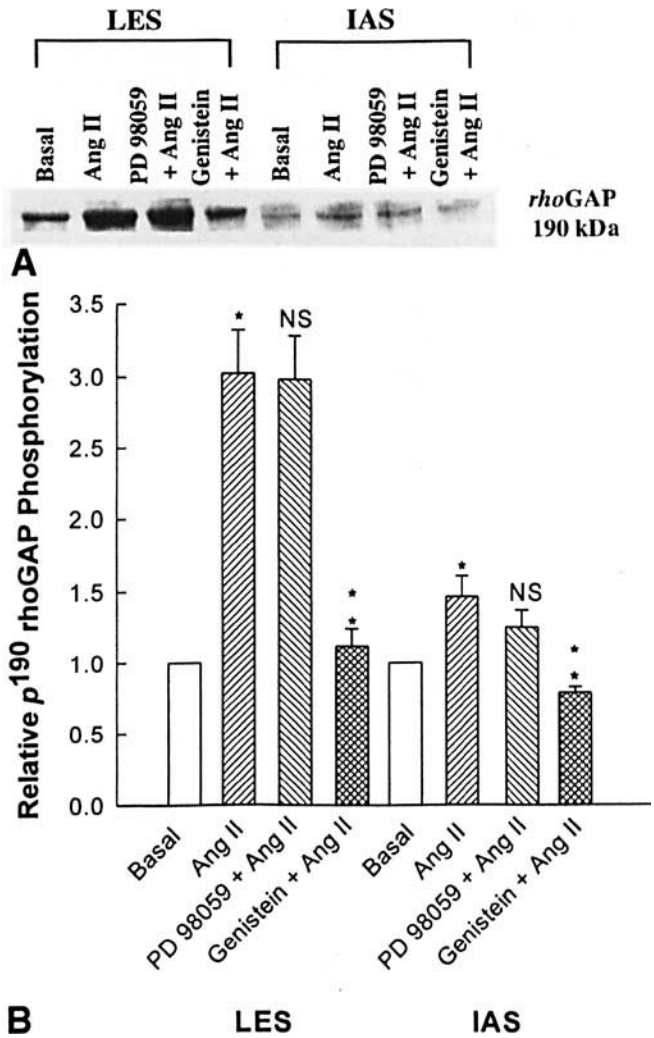

Figure 4. Effect of PD 98059 and genistein on tyrosine phosphorylation of p^{190} RhoGAP in LES and IAS smooth muscles contracted by Ang II. (A) The western blot. (B) The relative distribution of phosphorylated p^{190} RhoGAP by densitometric analyses. For these experiments, the tissue protein extracts were first immunoprecipitated with p^{190}RhoGAP antibody followed by immunoblotting with antiphosphotyrosine as described previously (49). Afterward, the membranes were stripped and reprobed with p^{190} RhoGAP antibody to determine the uniformity of loading of the specific protein. The controls correspond to basal levels of tyrosine phosphorylated p^{190} RhoGAP in these tissues (taken as 1). Note a significant increase in the levels of p^{190} RhoGAP tyrosine phosphorylation by Ang II (*$P < 0.05$; $n = 4$), that was not significantly modified by PD 98059, but was by genistein (**$P < 0.05$; $n = 4$).

ylation of p^{190} RhoGAP in the LES and IAS. Densitometric analyses of the western blots are shown in Figure 4B. PD 98059 (1×10^{-5} *M*) being a specific inhibitor of MEK (47, 50), had no significant effect on Ang II-mediated increase in the levels of tyrosine-phosphorylated p^{190} RhoGAP in the smooth muscles. Conversely, genistein, a general inhibitor of tyrosine kinases, caused a significant inhibition of the increase in the tyrosine phosphorylation of p^{190} RhoGAP in LES and IAS (**$P < 0.05$; $n = 4$; Fig. 4).

Effect of Antibodies to $G\alpha_{13\text{-}CT}$, $G\alpha_{13\text{-}CT}$ Peptide, and ARF antibody on Ang II-Induced Contraction of SMC from LES and IAS. Western blots using an anti-$G\alpha_{13\text{-}CT}$ antibody showed the presence of $G\alpha_{13}$ in rat LES and IAS smooth muscles (data not shown). Neither the anti-$G\alpha_{13\text{-}CT}$ antibody nor the $G\alpha_{13\text{-}CT}$ peptide had any significant effect on the percentage of shortening of the SMC from the LES and IAS and IAS\ in the basal state. In these experiments, Ang II in control experiments (in the presence of nonimmune IgG) caused contraction of permeabilized cells from LES and IAS to 17.4% ± 1.2% and 16.8% ± 1.7%, respectively. $G\alpha_{13\text{-}CT}$ antibody and $G\alpha_{13\text{-}CT}$ peptide significantly inhibited Ang II-induced contraction of permeabilized cells from LES (5.1% ± 0.6% and 4.5% ± 0.5%, respectively) and IAS (5.8% ± 0.8% and 6.1 ± 0.7%, respectively; *$P < 0.05$; $n = 4$; Fig. 5). ARF antibody caused a partial inhibition of Ang II-induced contraction of the SMC (Fig. 5). On the other hand, The combination of ARF and $G\alpha_{13\text{-}CT}$ antibodies lead to further attenuation of the contraction.

Effect of Y 27632 and Genistein on MLC_{20} Phosphorylation (MLC_{20}-P) in LES and IAS Smooth Muscles Contracted by Ang II. Western blots obtained by using antiphosphoserine monoclonal antibody showed that Ang II-induced contraction of LES and IAS was associated with an increase in the levels of serine phosphorylation of MLC_{20} (Fig. 6). Preincubation of LES and IAS smooth muscles with Y 27632 or genistein resulted in significant decrease in the levels of phosphorylation of MLC_{20}-P (**$P < 0.05$; $n = 4$). The results show that Ang II-induced contraction of smooth muscles involves a tyrosine phosphorylation signaling pathway(s), which activates ROK, a serine/threonine kinase.

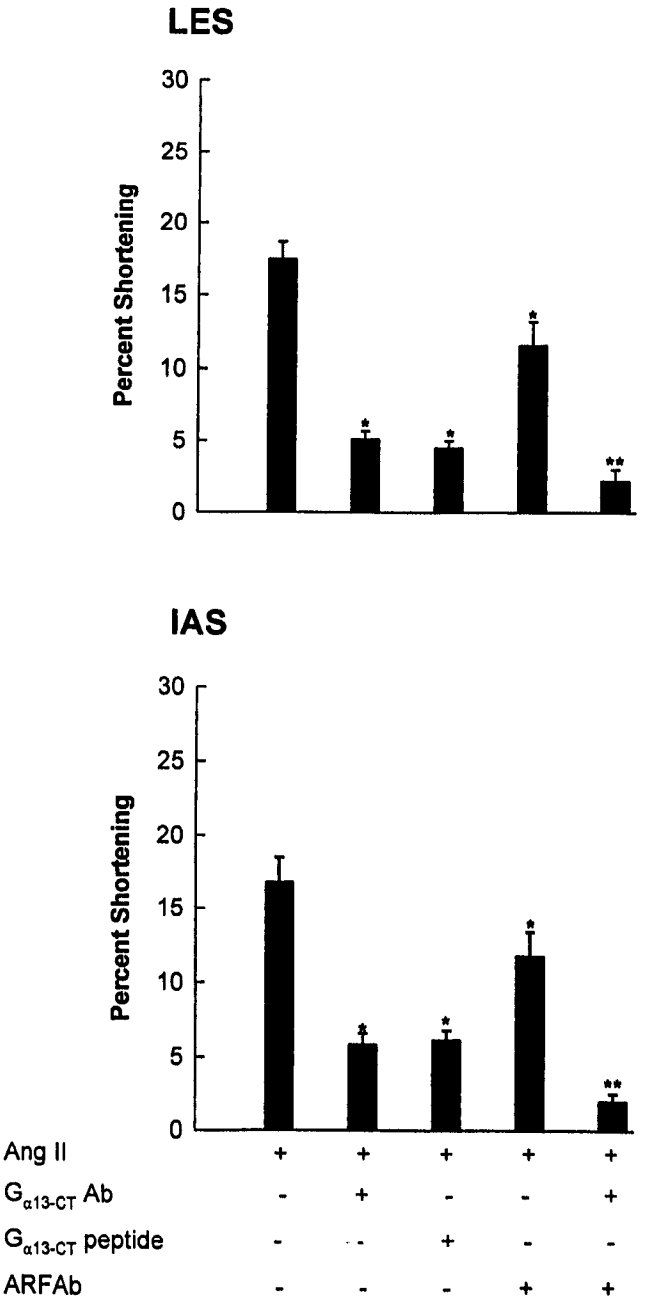

Figure 5. Effect of $G\alpha_{13\text{-}CT}$ antibody and $G\alpha_{13\text{-}CT}$ peptide (5 μg/ml) on Ang II-induced contraction of SMC from LES and IAS. Preliminary western blot studies confirmed the presence of $G\alpha_{13}$ in LES and IAS tissues. Permeabilized SMC from LES and IAS were incubated with $G\alpha_{13\text{-}CT}$ antibody, $G\alpha_{13\text{-}CT}$ peptide, or ARF antibody (5 μg/ml) for 1 hr at room temperature followed by Ang II. $G\alpha_{13\text{-}CT}$, $G\alpha_{13\text{-}CT}$ peptide, and ARF antibodies significantly inhibit Ang II-induced contraction of SMC from LES and IAS (*$P < 0.05$; $n = 4$). The combination of ARF and $G\alpha_{13\text{-}CT}$ antibodies causes further attenuation (**$P < 0.05$; $n = 4$).

Discussion

The studies suggest the role of monomeric G-proteins Rho and ARF as one of the pathways in Ang II-induced contraction of LES and IAS smooth muscles. Ang II-induced contraction of the SMC is inhibited by the ROK inhibitors, Y 27632 and HA 1077, at concentrations consistent with their specific inhibitory effects on ROK. Such concentrations tested in different systems may not cause significant inhibition of PKC, cAMP-dependent protein kinase, and MLCK (18, 19, 44). In addition, the C3 exozyme, a specific inhibitor of Rho (45, 46), attenuates Ang II-induced contraction of LES and IAS SMC. ROK is one of the effectors of Rho and is activated by its direct interaction with the small G-protein (15).

The participation of Rho signaling pathway after Ang II treatment in the LES and IAS smooth muscles is further supported by Ang II-induced increase in the levels of tyrosine phosphorylation of p^{190} RhoGAP. In addition, antibodies to $pp60^{c\text{-}src}$ and p^{190} RhoGAP and Hb A attenuate Ang

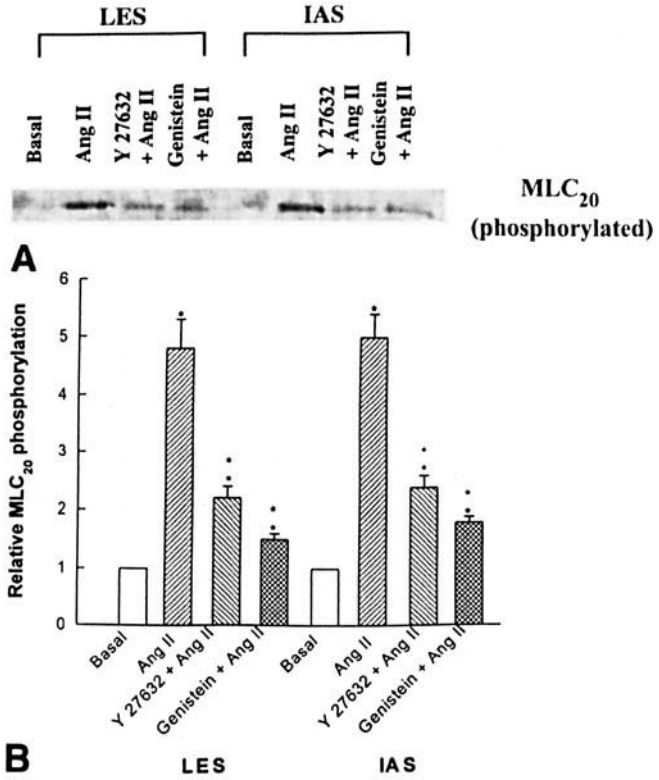

Figure 6. Effect of Y 27632 and genistein on Ang II-mediated increase in MLC_{20} phosphorylation in LES and IAS contracted by Ang II. (A) The western blot. (B) The relative distribution of phosphorylated MLC_{20} by densitometric analyses. For these experiments, the tissue protein extracts were first immunoprecipitated with MLC_{20} antibody followed by immunoblotting with antiphosphoserine as described previously (49). Afterward, the membranes were stripped and reprobed with MLC_{20} antibody to determine the uniformity of protein loading. The controls correspond to basal levels of tyrosine phosphorylated MLC_{20} in these tissues (taken as 1). Ang II caused a significant increase in MLC_{20}-P (*$P < 0.05$; $n = 4$), which were attenuated significantly by Y 27632 and genistein (**$P < 0.05$; $n = 4$).

II-induced contraction of the LES and IAS SMC. These results are in agreement with the earlier studies in different systems to suggest that p^{120} rasGAP and p^{190} RhoGAP are substrates for $pp60^{c\text{-}src}$ (48, 51). The data also suggest that Ang II-mediated stimulation of $pp60^{c\text{-}src}$ may be an upstream step in GPCR-mediated signaling mechanisms in Ang II effects. Activation of $pp60^{c\text{-}src}$ has been shown to be associated with the formation of a complex between p^{120} rasGAP and p^{190} RhoGAP (52). This coordinates ras- and Rho-mediated downstream signaling (53–55) in stimulated cells.

Ang II-mediated increase in the levels of tyrosine phosphorylation of p^{190} RhoGAP regulates the GTPase activity of Rho and may serve to coordinate downstream signaling by Rho. Findings by Nobes *et al.* (56) show that tyrphostin (an inhibitor of tyrosine phosphorylation) inhibits lysophosphatidic acid-mediated stress fiber formation without affecting stress fiber formation by Rho. Taken together, the results suggest that Rho serves to transmit signals from tyrosine kinases (e.g., $pp60^{c\text{-}src}$) to serine/threonine kinases (e.g., ROK).

The exact mechanism by which p^{190} RhoGAP antibody inhibits Ang II-induced contraction of LES and IAS SMC is not known. However, it is possible that the antibody used here, by an unidentified mechanism, affects the tyrosine phosphorylation of p^{190} RhoGAP, which attenuates the GTPase activity of Rho (57). This may result in the activation of Rho, followed by Rho kinase, the inhibition of MLC-phosphatase, an increase in MLC_{20}-phosphorylation, and finally, the contraction of smooth muscle. The findings are similar to $\alpha 6\beta 1$ integrin-induced invasiveness and tyrosine phosphorylation of p^{190} RhoGAP in adherent LOX melanoma cells that were attenuated by p^{190} RhoGAP antibody (49).

Present studies also show an involvement of $G\alpha_{13}$ in Ang II-induced contraction of LES and IAS SMC. A $G\alpha_{13\text{-}CT}$ antibody as well as the carboxyl terminus peptide ($G\alpha_{13\text{-}CT}$ peptide; used to produce the $G\alpha_{13\text{-}CT}$ antibody) inhibit Ang II-induced contraction of LES and IAS SMC. Additionally, we have observed significant levels of $G\alpha_{13}$ and $G\alpha_{12}$ in the rat tonic smooth muscles in the basal state (data not shown). The $G\alpha_{13\text{-}CT}$ antibody and $G\alpha_{13\text{-}CT}$ peptide have been shown previously to inhibit Ang II-mediated increase in $[Ca^{2+}]_i$ in rat portal vein myocytes (58). More recently, it has been shown that Rho guanine nucleotide exchange factors p^{115} RhoGEF (31, 32, 59) and PDZ-RhoGEF (60) form complexes with $G\alpha_{13}$, which in turn activate Rho.

In different smooth muscles examined, Ang II-induced contraction via AT_1 receptors involves multiple signal transduction systems such as release of intracellular Ca^{2+} ($[Ca^{2+}]_i$), Ca^{2+} influx, Ca^{2+}-calmodulin/MLCK, phospholipases, PKC, MAPKs, Janus kinases, tyrosine kinases, and small GTP-binding proteins (39, 61–63). AT_1 receptor activation leads to coupling with GTP-binding proteins such as G_q and $G_{12/13}$. Activation of Gq stimulates PLC-β to generate inositol-1,4,5-trisphospate (IP_3) and diacylglycerol (DAG). IP_3 causes the release of intracellular Ca^{2+} from IP_3-sensitive Ca^{2+} stores. This is followed by the influx of Ca^{2+} by the opening of voltage-sensitive Ca^{2+} channels, formation of Ca^{2+}-calmodulin (Ca^{2+}/CaM) complex, leading to the activation of Ca^{2+}/CaM-MLCK, causing smooth muscle contraction in the initial stages. DAG causes smooth muscle contraction by PKC activation. The initial surge of DAG is derived from phosphatidylinositol-4,5-bisphosphate, and sustained increased levels of DAG are provided by the activation of phospholipase D (PLD). The second phase of smooth muscle contraction with Ang II is primarily contributed by Ca^{2+} influx and activation of PLD. PLD causes the production of phosphatidic acid and DAG. DAG contributes to the prolonged activation of PKC and smooth muscle contraction (61). In addition to IP_3 formation and intracellular release of Ca^{2+}, tyrosine kinase-dependent increases in $[Ca^{2+}]_i$ have also been reported. PKC-independent contraction of Ang II-induced contraction of the SMC (that may involve protein kinase D or PKD) (64) has also been demonstrated (39).

Intracellular mechanisms that couple AT_1 receptors to PLD have been identified such as G-protein βγ subunits and associated $G_{\alpha 12}$ subunits. These G proteins activate PLD via src-dependent Rho pathways in vascular SMC (24, 65). The downstream signaling in this pathway may be PKC dependent or independent and may involve mobilization of $[Ca^{2+}]_i$ and Ca^{2+} influx. In addition, Rho A may regulate Ca^{2+} sensitivity via PKC/MAP kinase pathway or via PKC-mediated effect on MLC phosphatase (22, 66–70).

Present studies are in general agreement with those of Murthy *et al.* (19) and Cao *et al.* (20). These investigators showed that both $G\alpha_{13}$/Rho and ARF are involved in parallel in signal transduction, in CCK-induced contraction in the rabbit intestinal SMC, and in $PGF_{2\alpha}$-induced contraction in the cat esophageal SMC, respectively. Our data supports this thesis because antibodies to both $G\alpha_{13}$ and ARF caused attenuation of Ang II-mediated contraction of SMC. The combination of these antibodies causes further attenuation. Earlier studies (19, 20) also showed that actions of Rho and ARF are mediated via PLD/DAG/PKC-α pathways. Our preliminary findings that $G\alpha_{13}$ antibody causes inhibition of Rho kinase activity and that PLD inhibitor propranolol (in appropriate concentrations) causes inhibition of Ang II-induced contraction of the SMC provide further support to the hypothesis.

Different tyrosine kinases associated with GPCRs have been suggested to be involved in the upstream events following AT_1 receptor activation in different systems (16, 39, 71). It has been suggested that in addition to GEFs, activation of Rho kinase may be dependent on the activation of tyrosine kinase (56). Conversely, activation of Rho kinase may also affect indirectly tyrosine kinase downstream of Rho (72). Present data shows inhibition of Ang II-induced contraction of the SMC with the tyrosine kinase inhibitor. In addition, Rho kinase inhibitors Y 27632 and HA 1077, *C. botulinum* C3 exozyme, $pp60^{c\text{-}src}$, Hb A, and RhoGAP antibodies decrease phosphorylation of RhoGAP and MLC_{20}. These findings suggest that tyrosine kinase activation may be partly upstream. However, the exact nature of this tyrosine kinase is not known. The studies support the possibility of multiple intracellular pathways in the contraction of these smooth muscles because of a considerable crossover and interaction between different pathways. It is possible that Ca^{2+}-dependent pathways involving PLC and MLCK as well as the $G\alpha_{13}$/RhoA/ROK and ARF pathways and changes in cytoskeletal organization are required for efficient and sustained contraction of these smooth muscles after Ang II (19, 34, 73). The relative contribution of different pathways in the signal transduction of Ang II-induced contraction of LES and IAS smooth muscles and possible differences in the adjoining phasic smooth muscles remains to be determined.

The main points of this study are outlined in Figure 7. Ang II-induced contraction of the LES and IAS smooth

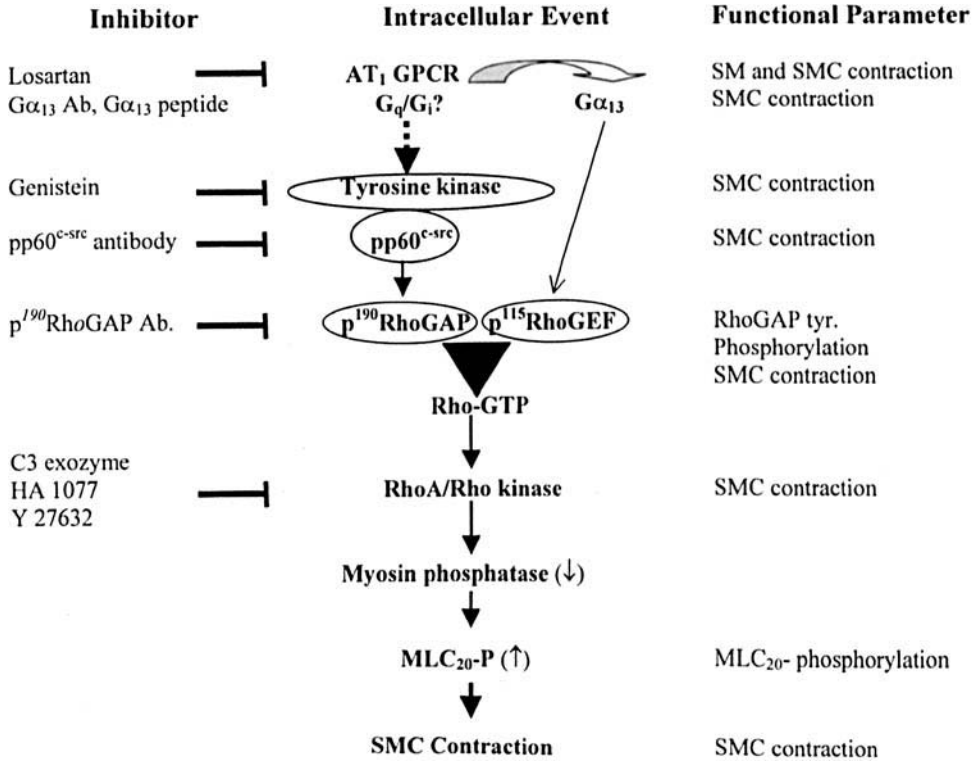

Figure 7. Hypothetical diagram to show Rho kinase signal transduction pathway involving Ang II-induced contraction of the sphincteric smooth muscle. Using AT_1 antagonist losartan, our earlier studies (8, 9) have shown that Ang II causes contraction of the SMC via activation of GPCR AT_1 receptor. AT_1 receptor activation may lead to the activation of a tyrosine kinase and complex formation with $pp60^{c\text{-}src}$. $pp60^{c\text{-}src}$-induced tyrosine phosphorylation of p^{190} RhoGAP and activation of Rho-guanine exchange factor (RhoGEF) lead to the formation of active form of Rho-GTP. In addition, $G\alpha_{13}$-mediated activation of Rho kinase may also be facilitated via p^{115} RhoGEF. p^{190} RhoGAP, and p^{115} RhoGEF complex formation, leading to Rho-GTP transformation leads to activation of RhoA/ROK. The latter may inhibit MLC phosphatase, leading to increased phosphorylation of MLC_{20}. The net effect of these events is an increase in the basal tone of the smooth muscle and contraction of the SMC. Different pharmacological tools used and parameters followed to test the events are listed in the figure. We have shown previously that multiple pathways including PKC, MLCK, and MAPK are involved in the smooth muscle contraction by Ang II. The role of G_q/PLCb/IP_3-DAG/Ca^{2+}-MLCK-PKC in relation to Ang II-induced contraction of the tonic smooth muscles is also of particular interest. For simplicity, these pathways have not been depicted in this diagram. The relative contribution and nature of exact interaction and interplay between different pathways in relation to RhoA/ROK activation in Ang II-induced contraction of IAS and LES smooth muscles remain to be determined.

muscles involves activation of Rho kinase downstream of $pp60^{c\text{-}src}$ and $G\alpha_{13}$. Activation of $G\alpha_{13}$ may also lead to the activation of Rho through p^{115} RhoGEF. Stimulation of p^{190} RhoGAP augments the GTPase activity of Rho and coordinates Rho-mediated downstream signaling. Ang II-mediated activation of ROK with the intermediary pathways such as PLD and PKC contribute to an increase in the levels of MLC_{20} phosphorylation, and in turn, to the smooth muscle contraction. Such information may be relevant for the increased understanding of intracellular pathways in the hypertensive sphincters responsible for certain gastrointestinal motility disorders.

1. Goyal RK, Sivarao DV. Functional anatomy and physiology of swallowing and esophageal motility. In: Castell DO, Richter JE, Eds. The Esophagus. Philadelphia: Lippincott, Williams & Wilkins, pp1–31, 1999.
2. Mittal RK, Balaban DH. The esophagogastric junction. N Engl J Med **336:**924–932, 1997.
3. Harnett KM, Cao WB, Kim N, Sohn UD, Rich H, Behar J, Biancani P. Signal transduction in esophageal and LES circular muscle contraction. Yale J Biol Med **72:**153–168, 1999.
4. Schiller LR. Fecal incontinence. In: Feldman M, Ed. Sleisenger and Fordrtran's Gastrointestinal and Liver Disease. Philadelphia: W.B. Saunders, pp160–173, 2000.
5. Culver PJ, Rattan S. Genesis of anal canal pressures in the opossum. Am J Physiol Gastrointest Liver Physiol **251:**G765–G771, 1986.
6. Goyal RK, Rattan S. Neurohumoral, hormonal, and drug receptors for the lower esophageal sphincter. Gastroenterology **74:**598–619, 1978.
7. Mukhopadhyay AK, Leavitt L. Evidence for an angiotensin receptor in esophageal smooth muscle of the opossum. Am J Physiol Endocrinol Metab **235:**E738–E742, 1978.
8. Rattan S, Fan Y-P, Puri RN. Comparison of angiotensin II (Ang II) effects in the internal anal sphincter (IAS) and lower esophageal sphincter smooth muscles. Life Sci **70:**2147–2164, 2002.
9. Fan Y-P, Puri RN, Rattan S. Animal model for angiotensin II effects in the internal anal sphincter smooth muscle: mechanism of action. Am J Physiol Gastrointest Liver Physiol **282:**G461–G469, 2002.
10. Puri RN, Fan Y-P, Rattan S. Role of $pp60^{c\text{-}src}$ and $p^{44/42}$ MAPK in ANG II-induced contraction of rat tonic gastrointestinal smooth muscles. Am J Physiol Gastrointest Liver Physiol **283:**G390–G399, 2002.
11. Boguski MS, McCormick F. Proteins regulating Ras and its relatives. Nature **366:**643–654, 1993.
12. Lamarche N, Hall A. GAPs for rho-related GTPases. Trends Genet **10:**436–440, 1994.
13. Matozaki T, Nakanishi N, Takai Y. Small G-protein networks: their crosstalk and signal cascades. Cell Signal **12:**515–524, 2000.
14. Ridley AJ. Rho: theme and variations. Curr Biol **6:**1256–1264, 1996.
15. Narumiya S. The small GTPase Rho: cellular functions and signal transduction. J Biochem **120:**215–228, 1996.
16. Pfitzer G, Arner A. Involvement of small GTPases in the regulation of smooth muscle contraction. Acta Physiol Scand **164:**449–456, 1998.
17. Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem **272:**12257–12260, 1997.
18. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature **389:**990–994, 1997.
19. Murthy KS, Zhou H, Grider JR, Makhlouf GM. Sequential activation of heterotrimeric and monomeric G proteins mediates PLD activity in smooth muscle. Am J Physiol Gastrointest Liver Physiol **280:**G381–G388, 2001.
20. Cao W, Harnett KM, Behar J, Biancani P. $PGF_{2\alpha}$-induced contraction of cat esophageal and lower esophageal sphincter circular smooth muscle. Am J Physiol Gastrointest Liver Physiol **283:**G282–G291, 2002.
21. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science **273:**245–248, 1996.
22. Horowitz A, Menice CB, Laporte R, Morgan KG. Mechanisms of smooth muscle contraction. Physiol Rev **76:**967–1003, 1996.
23. Iizuka K, Yoshii A, Samizo K, Tsukagoshi H, Ishizuka T, Dobashi K, Mori M. A major role for the Rho-associated coiled coil forming protein kinase in G-protein-mediated Ca2+ sensitization through inhibition of myosin phosphatase in rabbit trachea. Br J Pharmacol **128:**925–933, 1999.
24. Ushio-Fukai M, Alexander RW, Akers M, Lyons PR, Lassegue B, Griendling KK. Angiotensin II receptor coupling to phospholipase D is mediated by the $\beta\gamma$ subunits of heterotrimeric G proteins in vascular smooth muscle cells. Mol Pharmacol **55:**142–149, 1999.
25. Moss J, Vaughan M. Molecules in the ARF orbit. J Biol Chem **273:**21431–21434, 2002.
26. Dhanasekaran N, Dermott JM. Signaling by the G12 class of G proteins. Cell Signal **8:**235–245, 1996.
27. Moolenaar WH. Lysophosphatidic acid signalling. Curr Opin Cell Biol **7:**203–210, 1995.
28. Buhl AM, Johnson NL, Dhanasekaran N, Johnson GL. $G\alpha12$ and $G\alpha13$ stimulate Rho-dependent stress fiber formation and focal adhesion assembly. J Biol Chem **270:**24631–24634, 1995.
29. Sah VP, Seasholtz TM, Sagi SA, Brown JH. The role of Rho in G protein-coupled receptor signal transduction. Annu Rev Pharmacol Toxicol **40:**459–489, 2000.
30. Fields TA, Casey PJ. Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins. Biochem J **321:**561–571, 1997.
31. Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G, Sternweis PC. p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. Science **280:**2109–2111, 1998.
32. Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, Sternweis PC, Bollag G. Direct stimulation of the guanine nucleotide exchange activity of p^{115} RhoGEF by $G\alpha_{13}$. Science **280:**2112–2114, 1998.
33. Somlyo AP, Somlyo AV. Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J Physiol (Lond) **522:**177–185, 2000.
34. Wang P, Bitar KN. Rho A regulates sustained smooth muscle contraction through cytoskeletal reorganization of HSP27. Am J Physiol Gastrointest Liver Physiol **275:**G1454–G1462, 1998.
35. Harada T, Katsuragi T, Furukawa T. Release of acetylcholine mediated by cholecystokinin receptor from the guinea pig sphincter of Oddi. J Pharmacol Exp Ther **239:**554–558, 1986.
36. Stanciu C, Frasin M, Cijevschi C, Balan G, Pancu D, Haulica P. Effects of angiotensin on the human lower esophageal sphincter pressure. Med Intern **19:**359–361, 2002.
37. Catt KJ, Cain MD, Zimmet PZ, Cran E. Blood angiotensin II levels of normal and hypertensive subjects. Br Med J **1:**819–821, 1969.
38. Okamura T, Wang Y, Toda N. Local generation of angiotensin II in dog iris sphincter muscle. Exp Eye Res **55:**563–568, 1992.
39. Touyz RM, Schiffrin EL. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. Pharmacol Rev **52:**639–672, 2000.
40. Fan YP, Chakder S, Rattan S. Mechanism of action of cholera toxin on the opossum internal anal sphincter smooth muscle. Am J Physiol Gastrointest Liver Physiol **277:**G152–G160, 1999.
41. Chakder S, Bandyopadhyay A, Rattan S. Neuronal NOS gene expression in gastrointestinal myenteric neurons and smooth muscle cells. Am J Physiol Cell Physiol **273:**C1868–C1875, 1997.

42. Rattan S, Chakder S. Inhibitory effect of CO on internal anal sphincter: Heme oxygenase inhibitor inhibits NANC relaxation. Am J Physiol Gastrointest Liver Physiol **265**:G799–G804, 1993.
43. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685, 1970.
44. Schmidt M, Voss M, Weernink PAO, Wetzel J, Amano M, Kaibuchi K, Jakobs KH. A role for Rho-kinase in Rho-controlled phospholipase D stimulation by the m3 muscarinic acetylcholine receptor. J Biol Chem **274**:14648–14654, 1999.
45. Aktories K, Just I. *In vitro* ADP-ribosylation of Rho by bacterial ADP-ribosyltransferases. Methods Enzymol **256**:184–195, 1995.
46. Sekine A, Fujiwara M, Narumiya S. Asparagine residue in the *rho* gene product is the modification site for botulinum ADP-ribosyltransferase. J Biol Chem **264**:8602–8605, 1989.
47. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A **92**:7686–7689, 1995.
48. Schieffer B, Paxton WG, Chai Q, Marrero MB, Bernstein KE. Angiotensin II controls $p21^{ras}$ activity via $pp60^{c\text{-}src}$. J Biol Chem **271**: 10329–10333, 1996.
49. Nakahara H, Mueller SC, Nomizu M, Yamada Y, Yeh Y, Chen W-T. Activation of β1 integrin signaling stimulates tyrosine phosphorylation of p190RhoGAP and membrane-protrusive activities at invadopodia. J Biol Chem **273**:9–12, 1998.
50. Xiao ZL, Andrada MJ, Biancani P, Behar J. Reactive oxygen species (H_2O_2): effects on the gallbladder muscle of guinea pigs. Am J Physiol Gastrointest Liver Physiol **282**:G300–G306, 2002.
51. Marrero MB, Schieffer B, Paxton WG, Schieffer E, Bernstein KE. Electroporation of $pp60^{c\text{-}src}$ antibodies inhibits the angiotensin II activation of phospholipase C-γ1 in rat aortic smooth muscle cells. J Biol Chem **270**:15734–15738, 1995.
52. Ellis C, Moran M, McCormick F, Pawson T. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature **343**:377–381, 1990.
53. Cantor SB, Urano T, Feig LA. Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases. Mol Cell Biol **15**:4578–4584, 1995.
54. Nobes C, Hall A. Regulation and function of the Rho subfamily of small GTPases. Curr Opin Genet Dev **4**:77–81, 1994.
55. Settleman J, Albright CF, Foster LC, Weinberg RA. Association between GTPase activators for Rho and Ras. Nature **359**:153–154, 1992.
56. Nobes CD, Hawkins P, Stephens L, Hall A. Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J Cell Sci **108**:225–233, 1995.
57. Hu K-Q, Settleman J. Tandem SH2 binding sites mediate the RasGAP-RhoGAP interaction: a conformational mechanism for SH3 domain regulation. EMBO J **76**:473–483, 1997.
58. Macrez-Lepretre N, Kalkbrenner F, Morel J-L, Schultz G, Mironneau J. G protein heterotrimer Gα13β1γ3 couples the angiotensin AT1A receptor to increases in cytoplasmic Ca^{2+} in rat portal vein myocytes. J Biol Chem **272**:10095–10102, 1997.
59. Mao J, Yuan H, Xie W, Wu D. Guanine nucleotide exchange factor GEF115 specifically mediates activation of Rho and serum response factor by the G protein α subunit Gα13. Proc Natl Acad Sci U S A **95**:12973–12976, 1998.
60. Fukuhara S, Murga C, Zohar M, Igishi T, Gutkind JS. Novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J Biol Chem **273**:5868–5879, 1999.
61. Griendling KK, Ushio-Fukai M, Lassegue B, Alexander RW. Angiotensin II signaling in vascular smooth muscle. New concepts. Hypertension **29**:366–373, 1997.
62. Berk BC. Angiotensin II signal transduction in vascular smooth muscle: pathway activated by specific tyrosine kinases. J Am Soc Nephrol **10**:S62–S68, 1999.
63. Inagami T. Molecular biology and signaling of angiotensin receptors: an overview. J Am Soc Nephrol **10**:S2–S7, 1999.
64. Abedi H, Rozengurt E, Zachary I. Rapid activation of the novel serine/threonine protein kinase, protein kinase D by phorbol esters, angiotensin II, and PDGF-BB in vascular smooth muscle cells. FEBS Lett **427**:209–212, 1998.
65. Freeman EJ, Chisolm GM, Tallant EA. Role of calcium and protein kinase C in the activation of phospholipase D by angiotensin II in vascular smooth muscle cells. Arch Biochem Biophys **319**:84–92, 1995.
66. Hirata K, Kikuchi A, Sasaki T, Kuroda S, Kaibuchi K, Matsuura Y, Seki H, Saida K, Takai Y. Involvement of *rho* p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. J Biol Chem **267**:8719–8722, 1992.
67. Gong MC, Fujihara H, Somlyo AV, Somlyo AP. Translocation of *rhoA* associated with Ca^{2+} sensitization of rabbit smooth muscle. J Biol Chem **272**:10704–10709, 1997.
68. Kitazawa T, Eto M, Woodsome TP, Brautigan DL. Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. J Biol Chem **275**:9897–9900, 2000.
69. Li L, Eto M, Lee MR, Morita F, Yazawa M, Kitazawa T. Possible involvement of the novel CPI-17 protein in protein kinase C signal transduction of rabbit arterial smooth muscle. J Physiol (Lond) **508**:871–881, 1998.
70. Sward K, Dreja K, Susnjar M, Hellstrand P, Hartshorne DJ, Walsh MP. Inhibition of Rho-associated kinase blocks agonist-induced Ca^{2+} sensitization of myosin phosphorylation and force in guinea-pig ileum. J Physiol (Lond) **522**:33–49, 2000.
71. Di Salvo J, Nelson SR, Kaplan N. Protein tyrosine phosphorylation in smooth muscle: a potential coupling mechanism between receptor activation and intracellular calcium. Proc Soc Exp Biol Med **214**:285–301, 1997.
72. Ridley AJ, Hall A. Signal transduction pathways regulating Rho-mediated stress formation: requirement for a tyrosine kinase. EMBO J **13**:2600–2610, 1994.
73. Gohla A, Schultz G, Offermanns S. Role for G_{12}/G_{13} in agonist-induced vascular smooth muscle cell contraction. Circ Res **87**:221–227, 2000.