Rho A regulates sustained smooth muscle contraction through cytoskeletal reorganization of HSP27 

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Wang, Pinglang, and Khalil N. Bitar. Rho A regulates sustained smooth muscle contraction through cytoskeletal reorganization of HSP27. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1454–G1462, 1998.—The ras-related protein Rho p21 regulates various actin-dependent functions, including smooth muscle contraction. However, the precise mechanism of action of Rho p21 is still not clear. We report here that Rho A is a key regulator of agonist-induced contractile effects in rabbit colonic smooth muscle. Endothelin-1 and C2 ceramide were used. Both seem to activate phosphoinositol-3-kinase (PI 3-kinase) through G protein and pp60src, respectively. Immunoprecipitation and immunoblotting revealed one form of 21-kDa Rho A that translocated from the cytosol to the membrane in response to stimulation by either endothelin (10−7 M) or ceramide (10−7 M) (~30% increase at 30 s that was sustained at 4 min). The translocation of Rho A to the membrane was confirmed by immunostaining. The translocation of Rho A was inhibited by Clostridium botulinum C3 exoenzyme, which ADP ribosylates Rho A, but was not inhibited by the pp60src inhibitor herbimycin A or by the protein kinase C (PKC) inhibitor calphostin C, suggesting that Rho A may be upstream of pp60src and PKC or may belong to a different pathway than these proteins. Both ceramide- and endothelin-induced PI 3-kinase activation was inhibited by C3 exoenzyme pretreatment. However, the C3 exoenzyme inhibited endothelin- but not ceramide-induced mitogen-activated protein kinase phosphorylation, indicating that Rho regulates ceramide- and endothelin-induced contraction through different pathways. Furthermore, the dominant negative form of Rho (N19Rho) inhibited the actin binding protein, 27-kDa heat shock protein (HSP27), reorganization in response to ceramide and endothelin observed under confocal microscopy. 

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The low-molecular-weight GTP-binding protein Rho p21 is a member of the wide-spread superfamily of ras-related proteins, which can be divided into three subfamilies: Rho, Rac, and Cdc42 (45). These proteins function by utilizing a guanine nucleotide-binding and hydrolyzing cycle (5, 12). The Rho sequences were originally isolated from an Aplysia cDNA library (28) and were used subsequently to identify three homologues in mammals (Rho A, Rho B, and Rho C) (7) and the yeast Saccharomyces cerevisiae (29). The evidence to date indicates that Rho regulates the cytoskeletal system, particularly actin-dependent functions, such as cell motility (43), formation of stress fibers and focal adhesions (35), and smooth muscle contraction (15). The modes of activation and action of Rho p21 have not been identified. Similar to other G proteins, Rho p21 cycles between two interconvertible forms, GDP- and GTP-bound forms. In the cytoplasm, Rho is in the GDP-bound form, presumably the inactive form, complexed with Rho guanine nucleotide dissociation inhibitor (Rho-GDI). Rho receives upstream signals through their regulators and passes the signals to their downstream targets. It is activated by conversion to GTP-Rho by two possible mechanisms (45): 1) GDP-Rho first dissociates from Rho-GDI and is then activated by its stimulatory guanine nucleotide factor (GEF), resulting in the formation of GTP-Rho, or 2) GDP-Rho complexed with Rho-GDI is activated directly by a stimulatory GEF, resulting in the formation of GTP-Rho. It has been demonstrated that the Rho-Rho GDI system plays an important role in temporal and spatial determination of the actin cytoskeletal control (38). 

Stimulation of smooth muscle cells by specific agonists induces Ca2+ mobilization and activation of myosin light chain (MLC) kinase, which subsequently phosphorylates MLC and activates the myosin ATPase. The cascade of events described above leads to contraction of smooth muscle (41) and interaction of actin and myosin for stress fiber formation in nonmuscle cells (9). However, because the cytosolic concentration of Ca2+ is not always proportional to the extent of MLC phosphorylation and the force of contraction, it has been proposed that there may be an additional mechanism to regulate the Ca2+ sensitivity of both processes (6). Because agonists induce MLC phosphorylation and contraction in permeabilized smooth muscle at sub-maximal concentrations of Ca2+ in a GTP-dependent manner, a GTP-binding protein is thought to regulate the receptor-mediated sensitization of MLC phosphorylation to Ca2+ (24). The small GTPase Rho is implicated here in the enhancement of Ca2+ sensitivity of smooth muscle contraction by GTP (15). In permeabilized smooth muscle cells, the nonhydrolyzable GTP analog guanosine 5′-O-(3-thiotriphosphate) (GTPγS) increases MLC phosphorylation by inhibiting dephosphorylation of MLC presumably by activation of Rho (34). GTP-Rho then presumably binds to specific targets and thereby exerts its biological functions (33, 34). 

We have investigated how Rho A modulates agonist-induced signal transduction cascades in smooth muscle contraction and how it modulates actin binding proteins. We present evidence that Rho A is present in smooth muscle cells and that it is activated during ceramide- and endothelin-induced phosphoinositide 3-kinase (PI 3-kinase)-mediated sustained contraction. We also show that Rho A may regulate smooth muscle contraction through different pathways via reorganiza-
tion of the actin binding protein, 27-kDa heat shock protein (HSP27).

MATERIALS AND METHODS

Materials

The following reagents were purchased. Monodonal mouse anti-Rho A and polyclonal rabbit anti-Rho A IgG were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal mouse anti-PI 3-kinase NH₂-terminal-SH2 domain antibody was from Upstate Biotechnology (Lake Placid, NY); polyclonal rabbit phosphospecific p44/p42 mitogen-activated protein (MAP) kinase antibody was from New England Biolabs (Beverly, MA); FITC-conjugated Affinipure F(ab')₂ donkey anti-mouse IgG and lissamine rhodamine sulfonyl chloride (LRSC)-conjugated Affinipure F(ab')₂ sheep anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA); a monodonal mouse anti-HSP27 (IgG) used as was previously described (3); C₃ ceramide was from Matreya (Pleasant Gap, PA); and endothelin-1 was from Peninsula Laboratories (Belmont, CA). Clostridium botulinum C3 exoenzyme was from Biomol Research Laboratories (Plymouth Meeting, PA); collagenase type II was purchased from Worthington Biochemical (Freehold, NJ); herbimycin A was from Calbiochem (La Jolla, CA). Carphostin C was from Kamiya Biomedical (Thousand Oaks, CA). Wortmannin, soybean trypsin inhibitor (STI), poly-L-lysine, creatine phosphophate, creatine phosphokinase, and ATP were obtained from Sigma Chemical (St. Louis, MO); analytic silica gel 60% pre-coated glass TLC plates were obtained from Merck (Darmstadt, Germany); protein G-Sepharose was from Pharmacia Biotech (Uppsala, Sweden); [γ-32P]ATP and enhanced chemiluminescence (ECL) detection reagents were from Amersham (Beverly, MA); FITC-conjugated Affinipure F(ab')₂ donkey anti-mouse IgG and monoclonal anti-Rho A antibody (1:100 dilution) as primary antibody. The cell suspension was centrifuged down and resuspended in the cytosolic buffer containing antimycin A (10 µM), ATP (1.5 µM), and an ATP-regenerating system consisting of creatinene phosphate (5 µM) and creatine phosphokinase (10 U/ml). The permeabilized cells were allowed to rest in a 95% O₂-5% CO₂ environment before the experiments.

Immunoprecipitation and immunoblotting using monoclonal anti-Rho A antibody. Each sample (400–500 µg protein) obtained as described above was subjected to immunoprecipitation with monoclonal anti-Rho A antibody overnight at a ratio of 1:250. The protein G-Sepharose beads were then added and rocked for 2 h. The beads were washed in TBS twice and boiled in 2x Laemmli sample buffer with 2-mercaptoethanol. The samples were subjected to 12.5% SDS-PAGE and electroblotted onto nitrocellulose membrane. Immunoblotting was performed using mouse monoclonal anti-Rho A antibody (1:100 dilution) as primary antibody. The membrane was reacted with peroxidas-enzyme-conjugated goat anti-mouse IgG (1:3,000 dilution) for 1 h. The enzymes on the membrane were visualized with ECL substrates.

Measurement of PI 3-kinase activity. Whole cell lysates were obtained as described above. Equal amounts of protein were subjected to immunoprecipitation with anti-PI 3-kinase antibody (specific to the p85 subunit). The substrates were prepared by extracting the lipid mixture of 10 µg of phosphatidylinerine and 10 µg phosphoinositide in 1 ml methanol and chloroform (1:1), followed by 1 h sonication in 10 ml kinase assay buffer. The immunoprecipitates were then reacted with 10 ml lipid substrate mixture and 10 µl of 20 µM [γ-32P]ATP cocktail (2 µl [γ-32P]ATP with 8 µl of 104 µM ATP) in 15 µl kinase assay buffer for 10 min at 30°C. The reaction was then stopped by adding 100 µl 1 N HCl. The radioactive-labeled product Lα-phosphatidylinositol 4-monophosphate was extracted in 160 µl methanol and chloroform (1:1, vol/vol). The lipid extract was resolved by TLC using CHCl₃, MeOH, NH₄OH, and distilled H₂O (45:35:1.5:8.5, vol/vol). The TLC plates were dried and exposed to Kodak XOMAT LS film at 70°C for 1–2 days. Duplicates were also made for each reaction, as well as negative controls, by replacing the substrates with kinase assay buffer.
Measurement of contraction. Aliquots consisting of $2.5 \times 10^4$ cells in 0.5 ml of medium were added to 0.1 ml of a solution containing the test agents, which included agonists or combinations of inhibitors and agonists. The reaction was interrupted at 4 min by the addition of 0.1 ml of acrolein at a final concentration of 1%. Individual cell length was measured by computerized image micrometry. The average length of cells in the control state or after addition of test agents was obtained from 50 cells encountered randomly in successive microscopic fields. The contractile response is defined as the decrease in the average length of the 50 cells and is expressed as the absolute change or percent change from control length (3).

Cell culture and confocal imaging of HSP27 and Rho A. 

CELL CULTURE. The rabbit rectosigmoid was removed and washed in PBS with penicillin/streptomycin and ethanol twice. The mucosa and serosa were carefully removed, and the circular smooth muscle layer was washed three times in PBS with penicillin/streptomycin. The smooth muscle layer was digested with HEPES buffer containing 0.1% (wt/vol) collagenase (150 U/mg, Worthington CLS type II) at 37°C with 5% CO$_2$ for 2 h. At the end of the enzymatic incubation period, the tissue was washed in PBS with penicillin/streptomycin and DMEM supplemented with 10% FBS. The cell suspension was filtered by Nitek 500-µm mesh. The cell suspension was collected in the medium and transferred to six-well plates coated with collagen IV, which were then cultured in a humidified 5% CO$_2$ incubator for 7–14 days before they reached confluency.

COVERSLIP PREPARATION. Glass coverslips were washed in 70% ethanol for 30 min with gentle agitation. They were allowed to air dry, placed in six-well plates, and irradiated with ultraviolet light for 30 min. The coverslips were then coated with 0.5% (wt/vol) poly-l-lysine and allowed to air dry. The coverslips were placed in a six-well tissue culture plate. At the end of the second enzymatic digestion, the digested tissue was washed, titrated, and filtered as described previously. The dispersed cells were collected in DMEM with 10% FBS and transferred to the six-well tissue culture plate and allowed to settle for 2 days on the poly-l-lysine-coated coverslips in a humidified 5% CO$_2$ environment.

COVERSLIP PREPARATION. Glass coverslips were washed in PBS with penicillin/streptomycin and ethanol twice. The mucosa and serosa were carefully removed, and the circular smooth muscle layer was washed three times in PBS with penicillin/streptomycin. The smooth muscle layer was digested with HEPES buffer containing 0.1% (wt/vol) collagenase (150 U/mg, Worthington CLS type II) at 37°C with 5% CO$_2$ for 2 h. At the end of the enzymatic incubation period, the tissue was washed in PBS with penicillin/streptomycin and DMEM supplemented with 10% FBS. The cell suspension was filtered by Nitek 500-µm mesh. The cell suspension was collected in the medium and transferred to six-well plates coated with collagen IV, which were then cultured in a humidified 5% CO$_2$ incubator for 7–14 days before they reached confluency.

DUAL LABELING FOR RHO A AND HSP27. Dual labeling of RhoA and HSP27 was performed in either resting or contracted smooth muscle cells. A previously described protocol was used (46). After isolation, cells were cultured in DMEM with 10% FBS for 2 days on coverslips coated with poly-l-lysine. The cells were treated with C2 ceramide or endothelin-1, permeabilized, and fixed. Cells were incubated with the first secondary antibody, a 1:200 donkey anti-mouse IgG FITC, together with the second primary antibody, a monoclonal mouse anti-HSP27 (1:50), together with the second primary antibody, a polyclonal rabbit anti-Rho A (1:50), followed by three 10-min washes in PBS. Subsequently, the cells were incubated with the first secondary antibody, a 1:200 dilution of AffiniPure F(ab')2 donkey anti-mouse IgG FITC, together with the second primary antibody, a 1:100 dilution of AffiniPure F(ab')2 sheep anti-rabbit RITC, for 1 h, followed by three 10-min washes in PBS. Finally, the cells on the coverslip were mounted on a slide with DABCO mounting medium and sealed with Aqua Mount. The following controls were also performed: 1) FITC only, 2) RITC only, 3) FITC with first primary antibody, 4) RITC with first primary antibody, 5) FITC with second primary antibody, and 6) RITC with second primary antibody. The excitation for the fluorescent probes was as follows: FITC excitation at 492 nm and emission at 520 nm and LRSC excitation at 570 nm and emission at 590 nm. The confocal images were obtained on the Bio-Rad 600 confocal imaging system.

RESULTS 

C2 Ceramide- and Endothelin-1-Induced Activation and Translocation of RhoA in Colonic Smooth Muscle Cells

Both endothelin-1 and C2 ceramide can induce sustained smooth muscle contraction. Endothelin-1, a potent vasoconstrictor, acts through G protein (4), whereas C2 ceramide, a sphingomyelin breakdown product, seems to act through nonreceptor tyrosine kinase pp60c-src (19). Immunoprecipitation followed by immunoblotting using mouse monoclonal anti-Rho A antibody detected a single form of Rho A in the whole cell lysate of isolated smooth muscle cells in rabbit rectosigmoid, with a relative molecular mass of 21 kDa (Fig. 1). Rho GTPases are thought to be associated with Rho-GDI in the cytosol, as inactive forms, and therefore must translocate to the plasma membrane (where they would presumably meet a GEF) to be activated (13). Gong et al. (10) have demonstrated that 1) Rho A can translocate from the cytosolic to the particulate fractions on stimulation by agonists such as GTPγS and aluminum fluoride and 2) the association of RhoA with the plasma membrane may be required for its Ca$^{2+}$-sensitizing effect (11). To determine whether activation of Rho A is involved in contraction of isolated smooth muscle cells

![Fig. 1. Immunoblot detection of Rho A p21 in rabbit rectosigmoid smooth muscle cells, using monoclonal anti-Rho A antibody. Cell lysate (crude cytosolic fraction) was prepared from freshly isolated smooth muscle cells and subjected to immunoprecipitation with anti-Rho A antibody. The immunoprecipitate was then resolved by 12.5% SDS-PAGE and transferred to nitrocellulose membrane electrophoretically. The membrane was probed with monoclonal anti-Rho A antibody and visualized by enhanced chemiluminescence (ECL) reagents. Lane 1, Rho A p21 from HL-60 cell lysates. Lane 2, Rho A p21 from rabbit rectosigmoid smooth muscle cells.](http://ajpgi.physiology.org/)

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of the rabbit rectosigmoid induced by endothelin and ceramide, we stimulated the cells with C2 ceramide (10^-7 M) and endothelin-1 (10^-7 M) for different periods of time. The particulate fractions of smooth muscle cells were obtained by ultracentrifugation and extracted by 1% Triton X-100. On stimulation by endothelin or ceramide, Rho A did translocate from the cytosol to the membrane. At 30 s, ceramide and endothelin stimulation induced increases of ~30% in the intensity of the detected band of 21 kDa identified by immunological methods in the particulate fractions. The translocation was sustained at 4 min (Fig. 2). The translocation was also confirmed by immunostaining as described below. To further investigate the localization of Rho A, we performed immunofluorescent labeling of Rho A in isolated smooth muscle cells, followed by confocal microscopy (Fig. 3). Our data indicate that, at resting state (Fig. 3, elongated cell at left), Rho A is mainly distributed throughout the cell in the cytosol. In response to a 30-s treatment with C2 ceramide or endothelin-1, which induced the contraction and shortening of the cells (Fig. 3, contracted cell at right), the Rho A protein translocated from the cytosolic part and aligned itself along the membranes of contracted cells. It has been suggested that the recruitment of cytosolic proteins to the membrane is a pivotal component of several signaling systems, such as the raf-ras pathway (30) and protein kinase C (PKC) (17). Our immunoblotting data combined with immunostaining analysis suggested that Rho could serve as an important molecular switch controlling a signal transduction pathway by which the membrane receptors and the cytoskeletal systems are linked.

C3 Exoenzyme Inhibited Rho A Translocation to the Membrane and Decreased PI 3-Kinase Activity

Rho A is unique among the ras-related GTPases, since it is a specific substrate for ADP ribosylation catalyzed by the C3 exoenzyme from Clostridium botulinum (1, 32). C3 exoenzyme selectively modifies RhoA at the asparagine residue 41 and inhibits its biological function, presumably by interfering with its interaction with downstream targets (40). Because C3 exoenzyme is not cell permeable, we permeabilized the cells with saponin to examine the effects of C3 exoenzyme in the sustained contraction of smooth muscle cells induced by endothelin-1 and C2 ceramide. Preincubation of permeabilized smooth muscle cells with RhoA-specific C3 exoenzyme (2 ng/5,000,000 cells) for 20 min, followed by incubation with C2 ceramide or endothelin-1 for 30 s or 4 min at 37°C, resulted in decreased Rho A translocation to the membrane fractions (Fig. 4), which further confirmed that translocation is required for Rho A biological activity in smooth muscle contraction. We also examined PI 3-kinase activity, which in platelets requires the involvement of Rho in rapid thrombin-induced cytoskeletal reorganization (47). We found increased PI 3-kinase activity on ceramide and endothelin stimulation. However, after C3 exoenzyme treatment, it was found that C3 exoenzyme blocked the increased PI 3-kinase activity in response to both C2 ceramide and endothelin-1 (Fig. 5). These data suggest that Rho A in smooth muscle cells is an upstream regulator of PI 3-kinase activation.

C3 Exoenzyme Inhibits Colonic Smooth Muscle Contraction

We have previously shown that both C2 ceramide (10^-7 M) and endothelin-1 (10^-7 M) induce contraction...
The reaction was stopped by 1 N HCl. Lipid was exoenzyme, followed by ET-1 (10^{-7} M) for 30 s and 4 min. Soluble membrane fractions were obtained by ultracentrifugation and Triton X-100 extraction. RhoA bands in particulate fractions were detected by immunoblotting followed by immunoprecipitation. Lane 1, control relaxed cells; lanes 2 and 3, C2 ceramide (10^{-7} M) incubation for 30 s and 4 min, respectively; lanes 4 and 5, ET-1 (10^{-7} M) incubation for 30 s and 4 min, respectively; lanes 6 and 7, pretreatment of cells with C3 exoenzyme, followed by C2 ceramide (10^{-7} M) incubation for 30 s and 4 min, respectively; lanes 8 and 9, pretreatment of cells with C3 exoenzyme, followed by ET-1 (10^{-7} M) incubation for 30 s and 4 min, respectively.

Fig. 5. Inhibition of RhoA translocation by Clostridium botulinum C3 exoenzyme. Freshly isolated cells were permeabilized with saponin (75 µg/ml) and preincubated either with or without C3 exoenzyme (2 ng/5,000,000 cells) for 20 min. Cells were then stimulated with C2 ceramide (10^{-7} M) or ET-1 (10^{-7} M) for 30 s and 4 min. Soluble membrane fractions were obtained by ultracentrifugation and Triton X-100 extraction. RhoA bands in particulate fractions were detected by immunoblotting followed by immunoprecipitation. Lane 1, control relaxed cells; lanes 2 and 3, C2 ceramide (10^{-7} M) incubation for 30 s and 4 min, respectively; lanes 4 and 5, ET-1 (10^{-7} M) incubation for 30 s and 4 min, respectively; lanes 6 and 7, pretreatment of cells with C3 exoenzyme, followed by C2 ceramide (10^{-7} M) incubation for 30 s and 4 min, respectively; lanes 8 and 9, pretreatment of cells with C3 exoenzyme, followed by ET-1 (10^{-7} M) incubation for 30 s and 4 min, respectively.

of smooth muscle cells (4, 18). At 4 min, the smooth muscle cell length decreased by 36.14 ± 1.02% and by 40.1 ± 1.2% from control in response to C2 ceramide or endothelin-1. In cells preincubated with 1 ng C3 exoenzyme, the sustained contraction induced by ceramide was inhibited by 61.28 ± 9% and the contraction induced by endothelin was inhibited by 65.17 ± 3.8%. These data suggested that ceramide- and endothelin-induced colonic smooth muscle contraction is dependent on RhoA activation. However, ceramide- and endothelin-induced contractions could not be completely blocked by the RhoA inhibitor, indicating that other regulatory proteins may also be involved.

RhoA May Exert Its Effects on the Cytoskeleton via PI 3-Kinase Through Different Pathways

In an attempt to identify the pathways involved in RhoA activation, we used different antagonists to identify the possible upstream and downstream molecules related to RhoA function in cytoskeletal reorganization in smooth muscle contraction. Our data show that herbimycin A (3 × 10^{-6} M), a pp60^src kinase inhibitor, and calphostin C (10^{-6} M), a PKC inhibitor, did not affect RhoA activation induced by C2 ceramide and endothelin-1 (Fig. 6), indicating that RhoA may be upstream of pp60^src and PKC. We further examined the effect of C3 exoenzyme on MAP kinase, which seemed to be phosphorylated on stimulation with ceramide and endothelin (39). With C3 exoenzyme pretreatment (Fig. 7), C2 ceramide-induced MAP kinase phosphorylation was not inhibited, but endothelin-1-induced MAP kinase phosphorylation was inhibited by RhoA inactivation. Thus we propose that RhoA may regulate smooth muscle contraction in response to different agonists at least through diverged pathways via PI 3-kinase.

RhoA Activation Is Correlated with Redistribution of HSP27

We have previously reported that the low-molecular-weight HSP27, which has been shown to be an actin filament binding protein (31), is involved (3) and colocalizes with MAP kinase (46) in the PKC-mediated sustained contraction of rabbit colonic smooth muscle contraction. Here we investigated HSP27 fluorescence...
distribution under confocal microscopy in relation to Rho A activation in either resting or contracted rabbit rectosigmoid smooth muscle cells. On stimulation with C2 ceramide or endothelin-1, there was a clear translocation of Rho A to the cell membrane (Fig. 8, left), while HSP27 redistributed itself in the region of the surface membrane and formed traverse bands across the cells (Fig. 8, middle). The areas of strong colocalization on the cell edge (indicated by yellow dots) show that the pattern of colocalization is similar to that seen with redistribution of Rho A after contraction (Fig. 8, right). This result suggests that HSP27 translocates with Rho A during cell contraction and implicates the possible interaction between these two proteins. To further assess the relationship between Rho A and HSP27, we transfected the cells with the dominant negative form of Rho, N19Rho plasmid (Fig. 9), which inactivates Rho. HSP27 aligned itself along the axis of the cells, and there was no translocation of HSP27 that was seen in normal control cells (Fig. 10). These data suggest that Rho A may regulate actin structure through the PI 3-kinase-mediated HSP27 reorganization and translocation.

**DISCUSSION**

Ca2+ and MLC phosphorylation are key regulators of the dynamic reorganization of actin filaments. Because the contraction-to-Ca2+ ratio is not always proportional, the Ca2+- and calmodulin-dependent MLC kinase pathway cannot solely account for the Ca2+ sensitivity (6, 27, 42). In past years, evidence accumulated that the ras-related small GTP binding protein Rho is another important signaling element that mediates various actin-dependent cytoskeletal functions, including smooth muscle contraction (45). However, its roles in different signal transduction cascades may vary depending on cell type.

Rho has been shown to play pivotal roles in Ca2+ sensitization (23, 27). Several Rho targets have been
identified, including protein kinase N, Rho-associated kinase (Rho kinase), and the myosin-binding subunit of myosin phosphatase (22). It has been proposed that Rho activates Rho kinase, which in turn phosphorylates MLC by inhibiting myosin phosphatase (22, 23, 27). Here, we attempted to study Rho functions in the sustained contraction of rabbit rectosigmoid smooth muscle cells. Our data indicated that Rho plays an important role in the signal transduction modulating rabbit colon smooth muscle contractions, on stimulation by agonists such as endothelin-1, a known potent vasoconstrictor, as well as by C2 ceramide, which is a breakdown product of sphingomyelin hydrolysis.

Ceramide is an important regulatory molecule implicated in various biological processes in response to stress and cytokines. We have previously shown that the sustained contraction induced by the peptide agonist bombesin is accompanied by an increase in sphingolipid-derived ceramide. Ceramide produced in the cell acts as an intracellular messenger. Ceramide induces a sustained contraction of smooth muscle cells through a pathway that involves the activation of MAP kinase (39). Thus ceramide could be an important mediator of contraction and could account for the sustained contraction observed in circular smooth muscle cells from the rabbit rectosigmoid.

Endothelin-1 is a vasoconstrictor peptide originally derived from endothelial cells functioning as a local regulator of vascular tone and has been reported to possess a wide variety of other biological activities. Recent studies indicate the presence of endothelin-like immunoreactivity, endothelin-1 mRNA, and endothelin receptors in colon (20, 44). Evidence suggested that endothelin is a neuropeptide in the human intestine with binding sites on neural plexuses and mucosa, implying a role in the modulation of intestinal motility and secretion (20). Binding of endothelin-1 to its heterotrimeric G protein-coupled receptors stimulates various signaling cascades involving phospholipase C-\(\alpha\), phospholipase D, PKC, tyrosine kinases, Ca\(^{2+}\)- and calmodulin-dependent kinase, and Ras. It has also been shown that Rho activation is critical for the endothelin-1-induced nuclear signaling (21). Endothelin-1 could also cause translocation of Rho A to cell membrane in Swiss 3T3 fibroblasts (8). Furthermore, the Rho A inhibitor C3 exoenzyme could inhibit endothelin-induced cytoskeletal actin reorganization in cultured astrocytes (25) and tyrosine phosphorylation of p125 focal adhesion kinase and paxillin in Swiss 3T3 cells (36), suggesting the possible role of Rho in endothelin signaling pathways in smooth muscle contraction.

We have previously shown that endothelin-1 induces a sustained G protein-mediated contraction in smooth muscle cells of the colon (4) whereas C2 ceramide, a sphingolipid metabolite produced in smooth muscle cells, induces sustained contraction through activation of cytoplasmic tyrosine kinase of the Src family (19).
When stimulated with endothelin-1 or C2 ceramide, Rho A was observed evidently, by both Western blots and immunostaining data, to translocate from the cytosol to membrane. This translocation was blocked by the Rho A-specific inhibitor Clostridium botulinum C3 exoenzyme by ADP ribosylation of Rho A at the asparagine residue 41, perhaps by preventing Rho protein from interacting with its target molecule (40). It has been shown that, on receptor activation, Rho A translocates from the cytosol to the plasma membrane in certain cell types after the subsequent dynamic actin-cytoskeletal reorganization. When preincubating the cells with C3 exoenzyme, we observed that the contraction induced by ceramide and endothelin was inhibited. The data above strongly suggest that both endothelin-1 and C2 ceramide could exert their contractile effects via Rho A activation.

The possible interrelationship between Rho A and other serine/threonine kinases or tyrosine kinases is more complex. Our data show that the pp60c-src inhibitor herbimycin A was not able to inhibit Rho A translocation from the cytosol to the membrane, which suggested that Rho A activation by endothelin and ceramide is either independent or upstream of these tyrosine kinases. It has been suggested that Rho A is regulating Ca2+ sensitivity in smooth muscle via the PKC/MAP kinase pathway or through a PKC-mediated effect on MLC phosphatase (15, 16). After preincubation with the PKC inhibitor calphostin C, the data indicate that both ceramide- and endothelin-induced Rho A translocation is not inhibited, suggesting that Rho A is at least up stream of PKC. This view was also supported by the observation that Rho inhibitors block PKC translocation and activation in endothelial and epithelial cells, suggesting a Rho A requirement for PKC activation/translocation (14). We have also shown that both endothelin and ceramide can increase PI 3-kinase activity, with subsequent production of lipid to mediate actin cytoskeleton or interact with Rho A. It has previously been shown that Rho may regulate PI 3-kinase activity in Swiss 3T3 cells as well as platelets, suggesting that PI 3-kinase is downstream of RhoA (26, 47), although PI 3-kinase can be either upstream or downstream of the Rho family depending on the system (37). In our experiments, when smooth muscle cells were preincubated with Clostridium botulinum C3 exoenzyme, which selectively ADP ribosylates the Rho A in asparagine residue 41, the translocation of Rho A was inhibited, as was endothelin- and ceramide-induced PI 3-kinase activity. On the basis of these observations, we propose that ceramide and endothelin both activate Rho A, which in turn activates PI 3-kinase. However, it is of interest to understand whether Rho activates PI 3-kinase through a serine/threonine PKC pathway or via a tyrosine kinase pathway. Alternatively, ceramide and endothelin may also transduce their signals downstream of Rho A through different pathways to activate PI 3-kinase. It has been shown that ceramide and endothelin can phosphorylate p42 and p44 MAP kinase; our data also suggested that Rho A may regulate PI 3-kinase-mediated smooth muscle contraction induced by ceramide through pp60c-src, whereas endothelin directly activates MAP kinase cascade. The precise complex mechanism is yet to be determined.

Actin binding proteins play a key role in shaping the actin cytoskeleton. To further understand how Rho A protein affects actin filament dynamics induced by agonists in smooth muscle contraction, we assessed the correlation between Rho A and the low-molecular-weight HSP27 identified as an actin binding protein. HSP27 is expressed in a variety of tissues in the absence of stress and has been suggested to have a phosphorylation-activated homeostatic function at the actin cytoskeleton level. Phosphorylation of HSP27 has been shown to increase in response to diverse stimuli, including phorbol esters and Ca2+ ionophores. The degree of phosphorylation varies in response to different stimuli. HSP27 has also been suggested to be involved in contraction of intestinal smooth muscle. We have previously shown that HSP27 plays an integral role in the orientation or activation of the contractile machinery necessary to maintain a sustained contraction in rabbit gastrointestinal smooth muscle (46). HSP27 distribution during contraction of smooth muscle is not well understood. We previously reported that HSP27 could colocalize with MAP kinase during contraction. It has been reported that inhibition of Rho A by C3 exoenzyme could block endothelin-induced cytoskeletal actin reorganization in cultured astrocytes (25). In cells transiently transfected with the dominant negative Rho A, we observed that ceramide or endothelin-induced redistribution of HSP27 disappeared, which suggested that Rho A may exert its effects on cytoskeletal reorganization via HSP27. Moreover, the requirement of Rho A for HSP27 redistribution suggests the possible interaction between these cytoskeletal proteins in sustained smooth muscle contraction.

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REFERENCES


