HSP27 modulates agonist-induced association of translocated RhoA and PKC-\(\alpha\) in muscle cells of the colon

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Received 15 March 2001; accepted in final form 31 August 2001

Bitar, K. N., A. Ibitayo, and S. B. Patil. HSP27 modulates agonist-induced association of translocated RhoA and PKC-\(\alpha\) in muscle cells of the colon. *J Appl Physiol* 92: 41–49, 2002.—The recruitment of signal transduction molecules to the membrane is crucial for the efficient coupling of extracellular signals and contractile response. The trafficking is dynamic. We have investigated a possible cross talk between agonist-induced association of translocated RhoA and translocated protein kinase C-\(\alpha\) (PKC-\(\alpha\)) and a role for heat shock protein 27 (HSP27) in mediating this interaction. Immunoprecipitation with HSP27 monoclonal antibody followed by immunoblotting with either RhoA antibody or PKC-\(\alpha\) antibody indicated that acetylcholine induced associations of HSP27-RhoA and HSP27-PKC-\(\alpha\) in the membrane fraction but not in the cytosolic fraction. Immunoprecipitation with anti-RhoA monoclonal antibody followed by immunoblotting with PKC-\(\alpha\) antibody indicated that acetylcholine induced a significant complexing of RhoA-PKC-\(\alpha\) in the membrane fraction but not in the cytosolic fraction. In summary, the data indicate that agonist-induced contraction is associated with 1) association of translocated RhoA with HSP27 on the membrane, 2) association of translocated PKC-\(\alpha\) with HSP27 on the membrane, and 3) association of PKC-\(\alpha\) with RhoA on the membrane. The data suggest an important role for HSP27 in modulating a multiprotein complex that includes translocated RhoA and PKC-\(\alpha\).

cytoskeleton; contraction; signal transduction; heat shock protein 27; protein kinase C-\(\alpha\)

HEAT SHOCK PROTEIN 27 (HSP27) is a member of the mammalian small heat shock proteins family. HSP27 is expressed in a variety of tissues in the presence or absence of stress (16). HSP27 is relatively abundant in all types of cells (42), and it colocalizes with actin filaments in cardiac (28), skeletal (3, 41), and smooth muscle (5, 21). Evidence has shown that HSP27 is important in many cell functions, such as cell survival during stress, apoptosis mediated by Fas/APO-1 receptor, and microfilament organization in response to growth factor or stress, as well as smooth muscle contraction (5, 10). HSP27 has been shown to exhibit chaperone activity in vitro and modulate actin filament dynamics (14, 16). The physiological role of HSP27 is still undetermined. HSP27 becomes phosphorylated in response to heat shock and in response to different stimuli, such as cytokines, growth factors, and peptide hormones (27). HSP27 exists as both large oligomers that are hypothesized to have chaperone-like activity and as smaller oligomers that bind to and cap the barbed end of microfilaments and stabilize them (3, 41). Phosphorylation of HSP27 changes the actin cytoskeleton and actin-dependent events. Thus HSP27 may regulate and stabilize the cytoskeleton.

RhoA belongs to the superfamily of ras-related proteins (38). These proteins function by utilizing a guanine nucleotide-binding and -hydrolyzing cycle (7, 15). The evidence to date indicates that Rho regulates the cytoskeletal system, particularly actin-dependent functions, such as cell motility (37), formation of stress fibers and focal adhesions (30), and smooth muscle contraction (18, 36). However, the mode of action of RhoA in reorganization of the cytoskeleton has not been identified.

Recently, a model has been proposed in which Rho regulates myosin light chain (MLC) phosphorylation through its effectors, Rho-associated kinase (Rho kinase), and myosin-binding subunit (MBS) (2, 24, 36). GTP-bound Rho interacts with both Rho kinase and MBS of myosin phosphatase, resulting in activation of Rho kinase and translocation of MBS. Activated Rho kinase phosphorylates MBS, thereby inactivating myosin phosphatase (24). Concomitantly, Rho kinase phosphorylates MLC at the same site (Ser\(^{19}\)) that is phosphorylated by MLC kinase and could play a role in activation of myosin ATPase. Both events appear to be necessary for an increase in MLC phosphorylation, yet data suggest that Rho kinase appears to regulate MLC phosphorylation downstream of Rho in nonmuscle as well as in muscle cells (1).

Protein kinase C (PKC) isoforms are characterized by an NH\(_2\)-terminal regulatory domain containing binding sites for Ca\(^{2+}\), phosphatidylserine, and diacylglycerol; a small hinge region; and a COOH-terminal catalytic domain (29). PKC is regulated by multiple interdependent mechanisms, including enzymatic activation, translocation of the enzyme in response to activation, phosphorylation, and proteolysis. The PKC isoforms are divided into three families depending on differences in the regulatory domain (12). The isoforms...
exert different biological functions, yet in vitro the different PKC isoforms demonstrate little substrate specificity. Therefore, other mechanisms must be responsible for their differential effects. One possibility is directed translocation of PKC isoforms to their respective targets (39). One possible targeting mechanism for PKC is through its association with anchoring proteins that tether the enzyme to cellular structures.

Our laboratory (20) and others (44) have shown that HSP27 is phosphorylated in smooth muscle in response to contractile agonists and that HSP27 phosphorylation in gastrointestinal smooth muscle cells is inhibited by the PKC inhibitor calphostin C (20). Our laboratory has previously shown that PKC-α translocates to the membrane with stimulation with the contractile agonist ceramide (21). Our laboratory has also shown (40) that RhoA modulates agonist-induced signal transduction cascades in smooth muscle contraction and that when, examined under confocal microscopy, RhoA colocalizes on the membrane with the actin-binding protein HSP27. Therefore, HSP27 may be of importance in smooth muscle contraction. The studies of its structure-function relationship together with its interaction with other signal transduction pathways, namely RhoA and PKC will greatly complement our knowledge of signal transduction pathways mediating contraction in smooth muscle cells. We hypothesize that HSP27 mediates association of PKC-α and RhoA to the membrane during agonist-induced muscle contraction. We have attempted here to identify an agonist-induced association of HSP27 with translocated PKC-α and RhoA. The data suggest a mechanism by which PKC-α translocation is targeted to the membrane through its association with HSP27 and with RhoA on the membrane with stimulation with either ceramide or acetylcholine.

MATERIALS AND METHODS

Materials

C2 ceramide (0.1 μM) was from Matreya (Pleasant Gap, PA); collagenase type II was purchased from Worthington Biochemical (Freehold, NJ). Protein G-Sepharose was from Pharmacia Biotech (Uppsala, Sweden). Polyvinylidene fluoride (PVDF) membranes were from Bio-Rad (Hercules, CA); QiaGen Effectene transfection kit was from QiaGen (Valencia, CA); enhanced chemiluminescence detection reagents were from Amersham (Arlington Heights, IL). Monoclonal mouse anti-HSP27 antibody (2B4-123) was previously described (5). Monoclonal mouse anti-RhoA and polyclonal rabbit anti-RhoA IgG were from Santa Cruz Biotechnology (Santa Cruz, CA); herbinycin A was from Calbiochem (La Jolla, CA). Calphostin C was from Kamiya Biomedical (Thousand Oaks, CA). Wortmannin, soybean trypsin inhibitor, poly-l-lysine, creatinine phosphatase, creatinine phosphokinase, and ATP were obtained from Sigma Chemical (St. Louis, MO). All other reagents were purchased from Sigma Chemical.

Methods

Isolation of smooth muscle cells from rabbit rectosigmoid. Smooth muscle cells from rabbit rectosigmoid were isolated as described earlier (4). Briefly, the internal anal sphincter from anesthetized New Zealand White rabbits, consisting of the most distal 3 mm of the circular muscle layer, ending at the junction of skin and mucosa, was removed by sharp dissection. A 5-cm length of the rectosigmoid orad to the junction was dissected and digested to yield isolated smooth muscle cells. The tissue was incubated for two successive 1-h periods at 31°C in 15 ml of HEPES (pH 7.4) containing (in mM) 115 NaCl, 5.7 KCl, 2.0 KH2PO4, 24.6 HEPES, 1.9 CaCl2, 0.6 MgCl2, and 5.6 glucose containing 0.1% (wt/vol) collagenase (150 U/mg, Worthington CLS type II), 0.01 (wt/vol) soybean trypsin inhibitor, and 0.184 (wt/vol) DMEM. After the end of second enzymatic incubation period, the medium was filtered through 500-μm Nitex. The partially digested tissue left on the filter was washed four times with 10 ml of collagenase-free buffer solution. Tissue was then transferred into 15 ml of fresh collagenase-free buffer solution, and cells were gently dispersed. After a hemocytometric cell count, the harvested cells were resuspended in collagenase-free HEPES buffer (pH 7.4). Each rectosigmoid yielded 10–20 × 10^6 cells.

Particulate fractions. Isolated smooth muscle cells were counted on a hemocytometer and diluted with HEPES buffer as needed. Cells were then treated with agonists and/or antagonist for the indicated periods. After the treatment, the cells were washed twice with buffer A (in mM: 150 NaCl, 16 Na2HPO4, 4 NaH2PO4, and 1 sodium orthovanadate, pH 7.4) and sonicated in buffer B (1 mM Na3VO4, 1 mM NaF, 2 mM phosphomethanol-sulfonyl fluoride, 5 mM EDTA, 1 mM Na3MoO4, 1 mM dithiothreitol, 20 mM NaH2PO4, 20 mM NaHPO4, 20 mM Na4P2O7-10H2O, 50 μl/ml DNase-RNase, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml antipain, pH 7.4). The cell sonicates were centrifuged at 100,000 g for 60 min. The supernatant material from the high-speed centrifugation was collected as soluble cytosolic fraction. The pellet material was resuspended by sonication twice for 30 s in the lysis buffer plus 1% Triton X-100 and collected as soluble particulate fraction. The protein content was determined by using Bio-Rad protein assay reagent.

Immunoprecipitation and immunoblotting using monoclonal anti-RhoA antibody. Each sample (400–500 μg protein) obtained as described above was subjected to immunoprecipitation with 1:250 monoclonal anti-RhoA antibody overnight. Then the protein G-Sepharose beads were then added and rocked for 2 h. The beads were washed in Tris-buffered saline twice and boiled in 2X Laemmli sample buffer with 2-mercaptoethanol. The samples were subjected to 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Immunoblotting was performed using mouse monoclonal anti-RhoA antibody (1:100 dilution) as primary antibody. The membrane was reacted with peroxidase-conjugated goat anti-mouse IgG (1:3,000 dilution) for 1 h. The enzymes on the membrane were visualized with enhanced chemiluminescence substrates from Amersham.

Immunoprecipitation using monoclonal antibody. Smooth muscle cells were digested in HEPES buffer as needed. Cells were washed with buffer A (in mM: 150 NaCl, 16 Na2HPO4, 4 NaH2PO4, and 1 sodium orthovanadate, pH 7.4) containing (in mM: 150 NaCl, 16 Na2HPO4, 4 NaH2PO4, and 1 sodium orthovanadate, pH 7.4) PBS) containing 1 mM Na2VO4. The cells were then disrupted by sonication in buffer B (1 mM Na3VO4, 1 mM NaF, 2 mM phosphomethanol-sulfonyl fluoride, 5 mM EDTA, 1 mM Na3MoO4, 1 mM dithiothreitol, 20 mM NaH2PO4, 20 mM NaHPO4, 20 mM Na4P2O7-10H2O, 50 μl/ml DNase-RNase, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml antipain, pH 7.4). The cell sonicates were centrifuged for 15 min at 14,000 g. Protein G-Sepharose was washed twice with buffer B to make a 50% suspension. Lysate containing 200 μg protein in a total of 500 μl of buffer B was precleared with 50 μl of protein G-Sepharose bead slurry by rocking at 4°C for 30 min. The mixture was spun at 14,000 g for 5 min at 4°C, and ~2 μg of mouse monoclonal anti-RhoA...
antibody, mouse monoclonal anti-PKC-α antibody, or mouse monoclonal anti-HSP27 antibody were added to the resultant supernatant. The mixture was rocked at 4°C for 1 h followed by the addition of 50 μl of protein G-Sepharose bead slurry. The mixture was further rocked at 4°C for 2 h and spun at 14,000 g for 5 min, and the supernatant was aspirated off. The pellet was washed three times with buffer A and resuspended in 25 μl of 2× sample buffer and boiled for 5 min.

**SDS-PAGE and electrophoretic transfer.** For one-dimensional SDS-PAGE, the samples were mixed in an equal volume of 2× sample buffer [50 mM Tris, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, and 0.1% (wt/vol) bromophenol blue, pH 6.8]. The proteins were separated by 12.5 or 15% SDS-PAGE and transferred onto nitrocellulose or PVDF membranes. Proteins were identified by chemiluminescence. Autoradiography was performed on blots or dried gels using a PhosphorImager.

Western immunoblotting of lysates and immunoprecipitates. Lysates (80 μg) or immunoprecipitates of HSP27, RhoA, or PKC-α were size separated by SDS-PAGE and electrophoretically transferred to PVDF membranes. Immunoblotting was performed using a monoclonal anti-HSP27 antibody (1:5,000), a monoclonal anti-RhoA antibody (1:1,000), or a monoclonal anti-PKC-α antibody (1:1,000), as primary antibody. The membrane was reacted with peroxidase-conjugated goat anti-mouse IgG antibody (1:2,500 dilution) for 1 h at 24°C. The enzymes on the membrane were detected with luminescent substrates. As a negative control, blots were incubated in the secondary antibody only.

**Data analysis.** Bands were quantitated using a densitometer (model GS-700, Bio-Rad Laboratories), and band volumes (absorbance units × mm²) were calculated and expressed as a percentage of the total volume. Blotting data are within the linear range of detection for each antibody used.

**RESULTS**

Translocation of RhoA and association of translocated RhoA with HSP27 in the particulate fraction, in response to contraction induced by acetylcholine or by ceramide. Smooth muscle cells were stimulated with acetylcholine (0.1 μM) or with ceramide (0.1 μM) for 30 s or 4 min, and immunoprecipitates of HSP27 (anti-HSP27 antibody) from 500 μg of particulate fractions of cells were subjected to SDS-PAGE and Western blotting with anti-RhoA antibody (1:100; Fig. 1A). Stimulation with the contractile agonist ceramide (0.1 μM) resulted in a significant increase in the association of RhoA with HSP27 at 30 s and 4 min. Ceramide induced a 20.1 ± 0.6 and a 15.7 ± 1.8% increase in HSP27-RhoA association at 30 s and 4 min, respectively (Fig. 1B). Significant and sustained increases (12.9 ± 1.3%) in the association of HSP27 with RhoA were seen in cells stimulated with acetylcholine (0.1 μM) (Fig. 1B).
compared with control (8.8 ± 2.1%). In parallel experiments, there was no detectable association of HSP27 with RhoA in the cytosolic fraction of resting or unstimulated smooth muscle cells or with stimulation of the cells with either ceramide or acetylcholine (Fig. 1C), indicating that the association of the proteins is predominantly in the particulate fraction.

**Translocation of PKC-α and Association of Translocated PKC-α With HSP27 in the Particulate Fraction in Response to Contraction Induced by Acetylcholine or by Ceramide**

Cells were stimulated with ceramide (0.1 μM) or acetylcholine (0.1 μM) for 30 s or 4 min, and immunoprecipitates of HSP27 (anti-HSP27 antibody) from 500 μg of particulate fractions of cells were subjected to SDS-PAGE and Western blotted with anti-PKC-α antibody (1:200) (Fig. 2A). Stimulation with ceramide (0.1 μM) resulted in a significant increase in the association of HSP27 with PKC-α at 30 s and 4 min (11.5 ± 1.4 and 11.4 ± 0.7%) compared with control (8.8 ± 1.2%) (Fig. 2B). Significant and sustained increases (12.9 ± 1.3%) in the association of HSP27 with PKC-α were seen in cells stimulated with acetylcholine (0.1 μM) (Fig. 2B). In parallel experiments, there was no detectable association of HSP27 with PKC-α in the cytosolic fraction of resting/unstimulated smooth muscle cells or on stimulation of the cells with either ceramide or acetylcholine (Fig. 2C), indicating that the association of the proteins is predominantly in the particulate fraction.

**Association of Translocated RhoA With PKC-α in the Particulate Fraction in Response to Contraction by Acetylcholine or Ceramide**

Cells were stimulated with ceramide (0.1 μM) or acetylcholine (0.1 μM) for 30 s or 4 min, and immunoprecipitates of RhoA (anti-RhoA antibody) from 500 μg of particulate fractions were subjected to SDS-PAGE and Western blotted with anti-PKC-α antibody (1:200; Fig. 3A). Stimulation with acetylcholine (0.1 μM) resulted in a significant increase in the association of RhoA with PKC-α at 30 s and 4 min (13.2 ± 2.6 and 13.0 ± 2.2%, respectively) compared with control (7.3 ± 2.2%; Fig. 3B). Similar significant and sustained increases (12.9 ± 1.3) in the association of RhoA with PKC-α were seen in cells stimulated with ceramide (0.1 μM; Fig. 3B). Preincubation of smooth muscle cells with the PKC inhibitor calphostin C significantly inhibited acetylcholine-induced RhoA-PKC-α association at 30 s and 4 min (6.4 ± 1.03 and 8.9 ± 1.6%, respectively, P < 0.05), suggesting that PKC activation is necessary for its translocation to the membrane (Fig. 3C). In parallel experiments, there was a barely detectable background association of PKC-α with RhoA in the cytosolic fraction of resting/unstimulated smooth muscle cells or with stimulation of the cells with either ceramide or acetylcholine (Fig. 3, D and E), suggesting that the association of the proteins is predominantly in the particulate fraction.

Fig. 2. Coimmunoprecipitation of HSP27 with protein kinase C-α (PKC-α) in response to agonist stimulation in rabbit colon smooth muscle cells. Cells were either untreated or stimulated with ceramide (0.1 μM) or ACh (0.1 μM) for 30 s or 4 min. Immunoprecipitates of HSP27 (IP: anti-HSP27 antibody) from 500 μg of particulate fractions were subjected to SDS-PAGE and Western blotted with anti-PKC-α antibody (1:200; IB: anti-PKC-α antibody). A: a representative blot of 5 independent experiments showing an increase in the association of PKC-α with HSP27 with stimulation with either ceramide or ACh. B: stimulation with the contractile agonists ACh and ceramide (0.1 μM) resulted in a significant (P < 0.05) increase in the association of PKC-α with HSP27 at 30 s and 4 min compared with control. C: 500 μg of cytosolic fractions were immunoprecipitated with anti-HSP27 antibody and were subjected to SDS-PAGE and Western blotted with anti-PKC-α antibody (1:200). Representative of 3 blots showing there was no detectable association of HSP27 with PKC-α in the cytosolic fraction of resting unstimulated smooth muscle cells or with stimulation of the cells with ACh.
Fig. 3. Coimmunoprecipitation of RhoA with PKC-α in response to agonist stimulation in rabbit colon smooth muscle cells. Cells were either untreated or stimulated with ceramide (0.1 μM) or ACh (0.1 μM) for 30 s or 4 min. Immunoprecipitates of RhoA (IP: anti-RhoA antibody) from 500 μg of particulate fractions were subjected to SDS-PAGE and Western blotted with anti-PKC-α antibody (1:200; IB: anti-PKC-α antibody). A: representative blot of 3 independent experiments showing an increase in association of PKC-α with RhoA with ceramide or ACh stimulation. B: translocation and association of RhoA with PKC-α in the particulate fraction in response to contraction by ACh or ceramide. Cells were stimulated with ACh (0.1 μM) or ceramide (0.1 μM) for 30 s or 4 min in presence or absence of calphostin C, and immunoprecipitates of RhoA from 500 μg of particulate fractions were subjected to SDS-PAGE and Western blotted with anti-PKC-α antibody (1:200). Stimulation with ACh resulted in a significant (P < 0.05) increase in the association of RhoA with PKC-α at 30 s and 4 min. Similar significant (P < 0.05) and sustained increases in the association of RhoA with PKC-α were seen in cells stimulated with ceramide. C: calphostin C significantly inhibited RhoA-PKC-α association at 30 s and 4 min of ACh stimulation (6.4 ± 1.03 and 8.9 ± 1.6%, respectively; P < 0.05), suggesting that PKC activation is necessary for its translocation to the membrane. D: 500 μg of cytosolic fractions were immunoprecipitated with anti-PKC-α antibody and were subjected to SDS-PAGE and Western blotted with anti-RhoA antibody (1:200). Representative of 3 blots showing there was a barely detectable background association of PKC-α with RhoA in the cytosolic fraction of resting unstimulated smooth muscle cells or with stimulation of the cells with ACh. E: 500 μg of cytosolic fractions were immunoprecipitated with anti-RhoA antibody and were subjected to SDS-PAGE and Western blotted with anti-PKC-α antibody (1:200). Representative of 3 blots showing there was a barely detectable background association of PKC-α with RhoA in the cytosolic fraction of resting unstimulated smooth muscle cells or with stimulation of the cells with ACh.
Translocation of HSP27, RhoA, and PKC-α to the Particulate Fraction in Response to Contraction by Acetylcholine

To confirm that the increase in association is due to agonist-induced translocation of each of the proteins per se to the particulate fraction, cells were stimulated with acetylcholine (0.1 μM) for 30 s or 4 min, and immunoprecipitates of RhoA (anti-RhoA antibody) from 500 μg of particulate fractions were subjected to SDS-PAGE and Western blotted with anti-RhoA antibody (1:200). Sustained increase in the amount of RhoA translocated to the membrane and immunoprecipitated with RhoA antibody was observed (Fig. 4A). Similarly, particulate fractions from cells stimulated with acetylcholine (0.1 μM) for 30 s or 4 min were subjected to immunoprecipitation followed by Western blot with anti-PKC-α antibody. Significant increase in the amount of PKC translocated to the particulate fraction was evident (Fig. 4B). Translocation was inhibited when the cells were preincubated with calphostin C (Fig. 4B).

Furthermore, particulate fractions from cells treated with acetylcholine (0.1 μM) subjected to immunoprecipitation followed by Western blot with anti-HSP27 antibody (anti-HSP27 antibody, HSP27 antibody). Significant and sustained increase in the amount of HSP27 translocated to the membrane was evident (Fig. 4A). Similarly, particulate fractions from cells stimulated with acetylcholine (0.1 μM) for 30 s or 4 min were subjected to immunoprecipitation followed by Western blot with anti-HSP27 antibody (anti-HSP27 antibody, HSP27 antibody). Significant and sustained increase in the amount of HSP27 translocated to the particulate fraction was evident (Fig. 4B). Translocation was inhibited when the cells were preincubated with calphostin C (Fig. 4B).

DISCUSSION

Our laboratory has previously reported that preincubation of smooth muscle cells from the rabbit rectosigmoid with a monoclonal antibody to HSP27 inhibits PKC-mediated contraction (5). Our laboratory has also previously shown that RhoA may regulate smooth muscle contraction through cytoskeletal reorganization of HSP27 (40). The following physiological paradigm arises: “Does agonist-induced contraction result in phosphorylation of HSP27 and in an increase in association between HSP27 and PKC, which is paralleled by an increase in the association of PKC with RhoA?” Here, we have attempted to study the association of RhoA with PKC in agonist-induced sustained contraction of rabbit rectosigmoid smooth muscle cells. The data suggest that HSP27 may serve as a link between the two signal transduction pathways leading to contraction on stimulation by contractile agonists. Ca²⁺ and MLC phosphorylation are key regulators of dynamic actin filament reorganization. Because the contraction-to-Ca²⁺ ratio is not always proportional, the Ca²⁺/calmodulin-dependent MLC kinase pathway cannot solely account for the Ca²⁺-induced contraction (8, 26, 35). 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 (38). However, its roles in different signal transduction cascades may vary depending on cell type. RhoA has been shown to play pivotal roles in Ca²⁺ sensitization (24, 26). Several Rho targets have been identified, including protein kinase N, Rho kinase, and the MBS of myosin phosphatase (23). It has been proposed that Rho activates Rho kinase, which inhibits myosin phosphatase and results in increase in phosphorylated MLC (23, 24, 26, 36).

The possible interrelationship between RhoA and other serine/threonine kinases or tyrosine kinases is more complex. Our laboratory’s previous data (40) have shown that herbimycin A (pp60⁵⁰⁰ inhibitor) and genistein (tyrosine kinase inhibitor) did not inhibit RhoA translocation from the cytosol to the membrane.
The suggestion was that RhoA activation by endothelin and ceramide is either independent or upstream of these tyrosine kinases. It has been suggested that RhoA is regulating Ca\textsuperscript{2+} sensitivity in smooth muscle via the PKC and mitogen-activated protein kinase pathway or through a PKC-mediated effect on MLC phosphatase (18, 19). Thus activation of RhoA could be upstream of PKC-\(\alpha\), or the translocation of RhoA could be independent and parallel to the translocation and activation of PKC-\(\alpha\). Others have reported that in endothelial and epithelial cells, Rho inhibitors block PKC translocation and activation, suggesting RhoA requirement for PKC activation and/or translocation (17).

The accepted model of activation of PKC by lipids is that on binding of diacylglycerol in the presence of the phospholipid cofactor, a conformational change in PKC results in the removal of the pseudosubstrate from its binding site and in the activation of the enzyme. The cysteine-rich domain, the C\textsubscript{2} domain, and the pseudosubstrate domains are involved in phospholipid binding. The pseudosubstrate domain contributes to membrane binding. Membrane association is reflected in a shift in subcellular localization or translocation of cytosolic PKC to membrane compartments. This process is controlled by protein-protein interactions. In addition to binding to lipids, PKC can also bind with proteins via protein-protein interactions. These interactions play an important role in the localization and function of PKC isoforms. PKC-binding proteins bind PKC directly via a non-substrate-binding site, which may or may not be PKC substrates and may require cofactors for binding to PKC. PKC isoforms associate with cytoskeletal proteins (22). The interaction between PKCs and cytoskeletal proteins is in part isoform selective. It has been shown that PKC-\(\zeta\) binds to tubulin via the pseudosubstrate region (13). PKC-\(\epsilon\) binds to actin via an actin-binding site on the C1 region (31, 32). PKC-\(\beta\)II interacts with F actin, and this interaction protects PKC-\(\beta\)II from degradation and downregulation (6).

Actin-binding proteins play a key role in shaping the actin cytoskeleton. To further understand how RhoA protein affects actin filament dynamics induced by agonists in smooth muscle contraction, we assessed the correlation between RhoA and the low-molecular-weight heat shock protein HSP27 identified as an actin-binding protein. Our laboratory has 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 (43). HSP27 distribution during contraction of smooth muscle is not well understood. Recent data have shown that, in vascular smooth muscle, HSP27 redistributes from a cytosolic to a particulate fraction (9). It has been reported that inhibition of RhoA by C3 exoenzyme could block endothelin-induced cytoskeletal actin reorganization in cultured astrocytes (25). In cells transiently transfected with the dominant negative RhoA, our laboratory has observed (40) that ceramide- or endothelin-induced redistribution of HSP27 disappeared, which suggested that RhoA may exert its effect on cytoskeletal reorganization via HSP27. Our data (Fig. 4C) confirm an agonist-induced translocation of HSP27 to the particulate fraction, which could be a step in mediating the association of HSP27 with RhoA and with PKC-\(\alpha\).

There are several indications in other cell systems that suggest that RhoA- and PKC-mediated pathways interact. This was suggested to occur through the binding of RhoA to PKC-\(\alpha\) (11). Evidence that a cellular colocalization of PKC isozymes and RhoA and an apparent synergistic effect on cellular functions are very sketchy. Slater and co-workers (34) report that PKC-\(\alpha\) and RhoA could be coimmunoprecipitated from PC-12 cell lysates. The potentiating effect of RhoA was found to be specific for PKC-\(\alpha\). Slater et al. also reported that the membrane- or filamentous-actin associated PKC-\(\alpha\) activity, induced by phorbol ester, is further potentiated by RhoA. This may result from a distinct activating conformational change, suggesting that PKC- and RhoA-mediated signaling pathways may converge on a direct PKC-\(\alpha\)-RhoA interaction (33). Chang and co-workers (11) have suggested that Rho associates with PKC-\(\alpha\) in vivo and that membrane association and residues within the effector domain of Rho are required for maximal enhancement of activator protein-1 transscriptional activity, further underscoring the importance of the association of these proteins. Hippenstiel and co-workers (17) suggest that the interaction between these proteins require the cooperative interaction with other molecules. Similar results were reported in human epithelial cells.

Our data indicate that PKC-\(\alpha\) and RhoA do not associate in the cytosolic fractions of resting/unstimulated smooth muscle cells or in response to contractile agonists. PKC-\(\alpha\) and RhoA coimmunoprecipitate in the particulate fraction of colon smooth muscle cells in response to different contractile agonists. The literature does not provide evidence for a direct interrelationship between these two molecules. Data presented here show that both PKC-\(\alpha\) and RhoA coimmunoprecipitate with HSP27 in the particulate fraction. A possible explanation could be due to an association between Rho proteins and a PKC-adaptor protein. Our data suggest that HSP27 is a good candidate for such interaction. We propose a model by which HSP27 binds to PKC-\(\alpha\) and to RhoA on the membrane, as a result of activation of both PKC-\(\alpha\) and RhoA in contracting smooth muscle. With phosphorylation, HSP27 may bind to activated PKC\(\alpha\) and activated RhoA. Conversely, PKC-\(\alpha\), as it translocates to the membrane, would phosphorylate HSP27 and thus favors its association with RhoA. Thus HSP27 may function as a facilitator in thin-filament regulation of smooth muscle contraction.

We thank Mercy Pawar for technical assistance. This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1-DK-42876.
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