HSP27 in signal transduction and association with contractile proteins in smooth muscle cells

ADENIKE I. IBITAYO,[1] JEANETTE SLADICK,[1] SONY TUTEJA,[1] OTTO LOUIS-JACQUES,[1] HIROTAKA YAMADA,[1] GUY GROBLEWSKI,[2] MICHAEL WELSH,[3] AND KHALIL N. BITAR[1]

Departments of [1]Pediatrics, [2]Physiology, and [3]Anatomy and Cell Biology, University of Michigan Medical Center, Ann Arbor, Michigan 48109

Ibitayo, Adenike I., Jeanette Sladick, Sony Tuteja, Otto Louis-Jacques, Hirotaka Yamada, Guy Groblewski, Michael Welsh, and Khalil N. Bitar. HSP27 in signal transduction and association with contractile proteins in smooth muscle cells. *Am. J. Physiol.* 277 (*Gastrointest. Liver Physiol.* 40): G445–G454, 1999.—Sustained smooth muscle contraction is mediated by protein kinase C (PKC) through a signal transduction cascade leading to contraction. Heat-shock protein 27 (HSP27) appears to be the link between these two major events, i.e., signal transduction and sustained smooth muscle contraction. We have investigated the involvement of HSP27 in signal transduction and HSP27 association with contractile proteins (e.g., actin, myosin, tropomyosin, and caldesmon) resulting in sustained smooth muscle contraction. We have carried out confocal microscopy to investigate the cellular reorganization and colocalization of proteins and immunoprecipitation of HSP27 with actin, myosin, tropomyosin, and caldesmon as detected by sequential immunoblotting. Our results indicate that *1*) translocation of Raf-1 to the membrane when stimulated with ceramide is inhibited by vasoactive intestinal peptide (VIP), a relaxant neuropeptide; *2*) PKC-α and mitogen-activated protein kinase translocate and colocalize on the membrane in response to ceramide, and PKC-α translocation is inhibited by VIP; *3*) HSP27 colocalizes with actin when contraction occurs; and *4*) HSP27 immunoprecipitates with actin and with the contractile proteins myosin, tropomyosin, and caldesmon. We propose a model in which HSP27 is involved in sustained smooth muscle contraction and modulates the interaction of actin, myosin, tropomyosin, and caldesmon.

heat-shock protein 27; protein kinase C; colocalization; actin; immunoprecipitation; sustained smooth muscle contraction; Raf-1

HEAT-SHOCK protein 27 (HSP27) is a member of the mammalian heat-shock protein family. HSP27 is expressed in many cell types where it normally forms oligomers of 100–800 kDa. Under stress conditions, the formation of oligomers or aggregates of 2 million or more daltons occurs (16). One gene is responsible for expression of HSP27 protein in mammals, although nonexpressed pseudogenes also exist (26). HSP27 is relatively abundant in all types of tissues (7, 52), and it colocalizes with actin filaments in cardiac (36) and skeletal muscles (50). HSP27 exists as both large oligomers hypothesized to have chaperone-like activity (20) and an ability to act with glutathione to protect cells (37) and as smaller oligomers that are said to bind to and cap the barbed ends of microfilaments (38) and to stabilize them (32). A more specific role has been found for HSP27 in the regulation of actin-cytoskeletal dynamics (31). This role is based on the phosphorylation-dependent ability of HSP27 to modulate actin polymerization (6). However, the exact role of HSP27 in smooth muscle cell contraction is not known.

In smooth muscle cells, HSP27 appears to be the link between the signal transduction cascade and the contractile machinery. Numerous signal transduction pathways operate within smooth muscle cells. Thus cells can respond quickly and accurately to a number of extracellular signals. One of the prominent signal transduction proteins is the small GTPase Ras (19). The Ras-mediated signal transduction pathway links receptors at the plasma membrane to intracellular signaling cascades, involving the mitogen-activated protein (MAP) kinase cascades, which in turn leads to the activation and phosphorylation of several target proteins, e.g., HSP27 (41, 43). Two different contractile pathways have been identified in smooth muscle cells: *1*) a transient contraction, which is calmodulin-dependent, is mediated by inositol 1,4,5-trisphosphate-dependent calcium release, and is induced by agonists such as substance P and CCK-8 (8); and *2*) a prolonged contraction induced by agonists like bombesin and ceramide (9, 28) and mediated by extracellular calcium influx and by a protein kinase C (PKC)-dependent, calmodulin-independent pathway (4, 30, 40, 51). In smooth muscle cells of the rabbit colon, we have identified that HSP27 is phosphorylated in response to different agonists that induce PKC-mediated contraction (10, 27). Preincubation of smooth muscle cells with calphostin C, a PKC inhibitor, results in inhibition of contraction, inhibition of MAP kinase activation (42), and inhibition of phosphorylation of HSP27 (27).

In striated muscle cells, actin filaments are stable and the sliding of actin filaments as propelled by myosin cross bridges is the basic mechanism for contraction (21). The sliding filament cross-bridge model is also applicable to smooth muscle. However, actin filaments in smooth muscle may not be as stable as those in striated muscle (48). Some studies suggest that actin filaments in smooth muscle may be dynamic, like in nonmuscle cells (48). The initiation of smooth muscle contraction is attributed to an increase in intracellular calcium, leading to activation of myosin light-chain kinase (MLCK) and phosphorylation of the 20-kDa myosin light chain, yet less is known about the signaling events that lead to the maintenance of force. Sustained levels of intracellular calcium are not re-

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quired to maintain contraction, and the temporary relationship between myosin light-chain phosphorylation and tension development is variable. The mechanism of the greater force generation in smooth muscle is not well understood. It is possible that, on HSP27 reorganization, HSP27 binds to actin filaments, thus maintaining the cytoskeleton, which leads to easier generation of force.

Caldesmon is a major calmodulin and actin-binding protein that is found in muscle and nonmuscle cells (23, 45). Two isoforms of caldesmon can be distinguished, *h* caldesmon (~120 kDa) and *l* caldesmon (~70 kDa) (45). The interaction of *h* caldesmon with actin-tropomyosin switches the actomyosin ATPase activity off. Calmodulin binds to *h* caldesmon at activating calcium concentrations, modulating the interaction of *h* caldesmon with actin-tropomyosin, which results in activation of the myosin ATPase activity and thus contraction. In skeletal and cardiac muscle, tropomyosin plays a central role in the regulation of contraction by binding to the myosin-binding site on actin in the resting state (3). This effect is mediated by the troponin complex. In contrast, the physiological role of tropomyosin in smooth muscle cells is poorly understood because smooth muscle cells do not contain troponin.

We have examined the effect of ceramide-induced, PKC-mediated sustained contraction on actin reorganization of the cytoskeleton in smooth muscle cells. We have studied the association of HSP27 with actin and other actin binding proteins that are members of the contractile machinery in smooth muscle, namely, myosin, caldesmon, and tropomyosin. We have also tested the effect of 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7), an MLCK inhibitor, on agonist-induced contraction of smooth muscle cells. We report here that in smooth muscle cells of the rabbit rectosigmoid *1*) in response to stimulation by ceramide, Raf-1, PKC-α, and MAP kinase translocate (we have previously reported that MAP kinase translocates to the membrane on activation, and, at that point in time, it colocalizes with HSP27; Ref. 54); *2*) PKC-α colocalizes with MAP kinase when translocation occurs; *3*) in response to ceramide, HSP27 reorganizes into crossbands and colocalizes with actin; *4*) HSP27 immunoprecipitates with actin, myosin, tropomyosin, and caldesmon; and *5*) ceramide- and ACh-induced contractions are not entirely dependent on myosin-light chain phosphorylation. Taken together, the data suggest a role for HSP27 in PKC-mediated sustained smooth muscle contraction.

MATERIALS AND METHODS

Materials

Chemicals were purchased from the following sources: C2-ceramide from Matreya (Pleasant Gap, PA); DMEM, penicillin G sodium, streptomycin sulfate, and soybean trypsin inhibitor (SBTI) from GIBCO Laboratories (Grand Island, NY); and collagenase (CLS type II) from Worthington Biochemical (Freehold, NJ). The mouse monoclonal anti-HSP27 (IgG1) antibody (2B4-123) was previously described (10). Mouse monoclonal anti-actin antibody and mouse monoclonal anti-myosin antibody were from Chemicon International (Temecula, CA); mouse monoclonal anti-tropomyosin antibody and mouse monoclonal anti-caldesmon antibody were from Sigma (St. Louis, MO); anti-Raf-1 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PKC isoform-specific antibodies (polyclonal) were from PanVera (Madison, WI); anti-mouse IgG-FITC and anti-mouse IgG-lissamine rhodamine sulfonyl chloride (LRSC) were from Jackson Immunoresearch Laboratories (West Grove, PA); goat anti-mouse IgG (H+L)-horseradish peroxidase conjugate and protein assay reagent were from Bio-Rad Laboratories (Hercules, CA); and goat anti-rabbit IgG (H+L) peroxidase-labeled antibody was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Protein G-Sepharose was from Pharmacia Biotech (Uppsala, Sweden), and enhanced chemiluminescence detection reagents were from Amersham (Arlington Heights, IL). All other reagents were purchased from Sigma.

Methods

Confocal imaging. CELL PREPARATION. Rabbit colons (5 cm orad to the internal anal sphincter) were dissected, immediately rinsed in ~20 ml of ice-cold PBS with penicillin G sodium (100 U/ml)-streptomycin sulfate (100 μg/ml), and placed in the same solution that had been previously gassed with 95% O_2-5% CO_2. Under a vertical laminar flow condition, the mucosa was removed and the tissue was rinsed three times in PBS with antibiotics. The tissue was then sliced into small pieces in a new petri dish containing PBS with antibiotics and then placed in a 25-cm^3 tissue culture flask with 10 ml of sterile digestion mixture (1 mg/ml collagenase, 50 U/ml penicillin, and 50 μg/ml streptomycin in Hanks' balanced salt solution). Digestion was carried out for two successive 1-h periods at 37°C in a 5% CO_2 atmosphere, with occasional shaking. After digestion, the tissue was rinsed thoroughly with PBS containing antibiotics over a 100-μm sterile mesh and then transferred to a new 25-cm^3 tissue culture flask containing 10 ml of DMEM-F12 medium supplemented with N_2 (12). Cells were harvested by filtering the solution through a fresh 100-μm sterile mesh.

COVERSLIP PREPARATION. Glass coverslips were washed in 95% ethanol for 30 min with gentle agitation. They were allowed to air dry and placed in a 16-well tissue culture plate and ultraviolet irradiated for 30 min. Cells were added and allowed to settle overnight in a humidified 5% CO_2 environment.

CELL FIXATION. Cells were either untreated or treated with ceramide (10^{-6} M), and the reaction was stopped by removing the medium and adding 3.5% formaldehyde in PBS. Cells were fixed for 10 min. The fixative was removed, and the cells were washed twice with 100 mM glycine solution, pH 7.4, for 5 min, followed by one wash with PBS. The cells were then permeabilized by adding 3 ml of the permeabilization solution [0.1 ml Triton X-100, 90 ml distilled water, and 10 ml PBS (10×)] to each coverslip for 10 min. After permeabilization, the cells were rinsed three times with 3-ml aliquots of PBS.

Labeling with Raf-1 monoclonal antibody. Cells were incubated for 1 h with blocking antibody [AffiniPure F(ab′)$_2$ sheep anti-mouse IgG] followed by three 10-min washes in antibody wash solution with gentle agitation. Subsequently, the cells were incubated for 1 h in the primary antibody, a mouse monoclonal anti-Raf-1 IgG at 0.033 mg/ml, followed by three 10-min washes in antibody wash solution with gentle agitation. This step was followed by incubation of the cells for 1 h in the secondary antibody [AffiniPure F(ab′)$_2$ donkey anti-mouse IgG-rhodamine LRSC] followed by three 10-min washes in antibody wash solution with gentle agitation. Finally, the cells on the coverslip were mounted on a slide with 1,4-diazabicyclo(2,2,2)octane (DABCO) mounting medium and sealed with Aquamount.

Control slides were made by incubating cells in the secondary antibody only. The excitation parameters for the fluores-

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cent probes were as follows: LRSC excitation at 570 nm and emission at 590 nm.

Dual labeling with two antibodies for HSP27 and actin or PKC-α and MAP kinase. Cells were incubated for 1 h with the first primary antibody, a mouse monoclonal anti-smooth muscle actin (IgG) at 0.033 mg/ml, followed by three 10-min washes in antibody wash solution, with gentle agitation. The cells were incubated for 1 h with the first secondary antibody, 1/30 dilution [an AffiniPure $F(ab')_2$ donkey anti-mouse IgG-FITC]. The cells were then washed three times for 10 min each in antibody wash solution with gentle agitation. Cells were incubated for 1 h with blocking antibody [AffiniPure $F(ab')_2$ sheep anti-mouse IgG], followed by three 10-min washes in antibody wash solution with gentle agitation. Subsequently, the cells were incubated for 1 h with the second primary antibody, a mouse monoclonal anti-HSP27 (IgG1) at 0.033 mg/ml, followed by three 10-min washes in antibody wash solution with gentle agitation. This procedure was followed by incubation of the cells for 1 h with the second secondary antibody [AffiniPure F(ab′)2 donkey anti-mouse IgG-rhodamine] and then by three 10-min washes in antibody wash solution with gentle agitation. Finally, the cells on the coverslip were mounted on a slide with DABCO mounting medium and sealed with Aquamount. Labeled cells were visualized using a laser-scanning confocal microscope (MRC-600) equipped with a Nikon oil-immersion objective (×60/1: 40). The images were obtained at focal planes at 0.4 µm distances, analyzed using the software programs XV3 and Adobe Photoshop, and photographed with a Polaroid CI-5000 film recorder. A similar procedure was followed for dual labeling for PKC-α and MAP kinase.

Control slides were made using the following protocol: *1*) antibody wash solution, first secondary antibody, *2*) antibody wash solution, second secondary antibody, *3*) first primary antibody, first secondary antibody, *4*) second primary antibody, second secondary antibody, *5*) first primary antibody, first secondary antibody, blocking antibody buffer, second secondary antibody, and *6*) first primary antibody, antibody wash solution, block, antibody wash solution, second secondary antibody. The excitation parameters for the fluorescent probes were as follows: FITC excitation at 492 nm and emission at 520 nm and LRSC excitation at 570 nm and emission at 590 nm.

Isolation of smooth muscle cells from rabbit rectosigmoid. The internal anal sphincter, consisting of the distal-most 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. Cells were isolated as previously described (9, 11, 42). Circular muscle tissue was incubated for two successive 1-h periods at 31°C in 15 ml of HEPES buffer (pH 7.4) (in mM): 115 NaCl, 5.7 KCl, 2.0 KH_2PO_4, 24.6 HEPES, 1.9 $CaCl_2$, 0.6 $MgCl_2$, 5.6 glucose containing 0.1% (wt/vol) collagenase, 0.01% (wt/vol) SBTI, and 0.184% (wt/vol) DMEM. At the end of the second enzymatic incubation period, the medium was filtered through a 500-µm Nitex filter. 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) and diluted as needed. Each rectosigmoid yielded 10–20 × 10^6 cells.

Immunoprecipitation using a monoclonal antibody. Isolated smooth muscle cells were diluted in HEPES buffer as needed. Untreated cells were washed with *buffer A* [in mM: 150 NaCl, 16 Na_2HPO_4, 4 NaH_2PO_4, pH 7.4 (PBS), containing 1 mM Na_3VO_4]. The cells were then disrupted by sonication in *buffer B* [1 mM Na_3VO_4, 1 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 1 mM Na_4MoO_4, 1 mM dithiothreitol, 20 mM NaH_2PO_4, 20 mM Na_2HPO_4, 20 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 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] and centrifuged for 15 min at 14,000 *g*. Protein G-Sepharose was washed two times with *buffer B* to make a 50% suspension. Lysate containing 200 µg of 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 1–2 µg of 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 3 times with *buffer A* and resuspended in 25 µl of 2× sample buffer and boiled for 5 min.

Western immunoblotting of immunoprecipitates. Immunoprecipitates were subjected to SDS-PAGE and electrophoretically transferred to PMSF membrane. Sequential immunoblotting was performed using a monoclonal anti-HSP27 antibody (1:5,000), a monoclonal anti-actin antibody (1:1,000), a monoclonal anti-myosin antibody (1:2,000), a monoclonal anti-tropomyosin antibody (1:1,000), and a monoclonal anti-caldesmon antibody (1:100), as primary antibody. Then, the membrane was reacted with peroxidase-conjugated goat anti-mouse IgG antibody (1:2,500 dilution) for 1 h at 24°C.

Each blot was sequentially reprobed with antibodies to the other members of the group. It was not necessary to strip membranes of already bound primary and secondary antibodies before reprobing, since there is no overlap in the molecular weights of all five proteins (HSP27, actin, myosin, tropomyosin, and caldesmon) coimmunoprecipitating. The enzymes on the membrane were detected with luminescent substrates. As a negative control, blots were incubated in the secondary antibody only.

Western immunoblotting using anti-PKC isoform-specific antibodies. Particulate fractions were run on SDS gels as previously described (49) and Western blotted with the anti-PKC isoform-specific polyclonal antibodies (α, βII, and γ) (1:200). The membrane was then reacted with a peroxidase-conjugated goat anti-rabbit antibody (1:10,000) for 1 h at 24°C. The enzymes on the membrane were detected with luminescent substrates.

Measurement of contraction. Aliquots consisting of 2.5 × 10^4 cells in 0.5 ml of medium were added to 0.1 ml of a solution containing ceramide (10^{-6} M) or ACh (10^{-7} M) with or without prior incubation with ML-7 (10^{-5} M). The reaction was allowed to proceed for 30 s or 4 min and stopped by the addition of 0.1 ml of acrolein at a final concentration of 1% (vol/vol). 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 the percent change from control length (9).

RESULTS

Cytoskeletal Reorganization of Raf-1 on Contraction of Smooth Muscle Cells

Using the confocal microscopy technique, we have investigated the reorganization of Raf-1 inside the cell when stimulated with ceramide (10^{-6} M). When contraction in response to stimulation by ceramide (10^{-6} M)

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occurs, Raf-1 appears to translocate predominantly to the region of the surface membrane (Fig. 1, *left cell*). Raf-1 translocation to the surface membrane was inhibited by preincubating the cells with vasoactive intestinal peptide (VIP; 10^{-6} M) for 10 min before stimulation with ceramide (10^{-6} M) (Fig. 1, *right cell*).

Translocation of PKC-α and Colocalization With MAP Kinase on Contraction of Smooth Muscle Cells

In the resting state, PKC-α is distributed throughout the cell (Fig. 2, elongated cell on *left*). However, when activated, PKC-α translocates to the membrane in response to stimulation by ceramide (10^{-6} M) (Fig. 2, *middle cell*). We have previously shown that ceramide induced activation of MAP kinase (28). The areas of colocalization of PKC-α with MAP kinase on translocation in response to stimulation by ceramide (10^{-6} M) appear to be limited mostly to the cell membrane (Fig. 2, *right cell*). The activation of PKC-α by contractile agonists that resulted in PKC-α translocation to the membrane was further confirmed by subcellular fractionation, followed by immunoblotting of proteins; ceramide (10^{-6} M) induced a translocation of PKC-α to the particulate (membrane) fraction (Fig. 3*A*, *lane 2*). PKC-α translocation to the membrane was inhibited by VIP (10^{-6} M) (Fig. 3*A*, *lanes 3* and *4*). Conversely, PKC-γ did not translocate to the membrane in response to ceramide stimulation (Fig. 3*B*). Ceramide also failed to induce PKC-βII translocation to the membrane (data not shown).

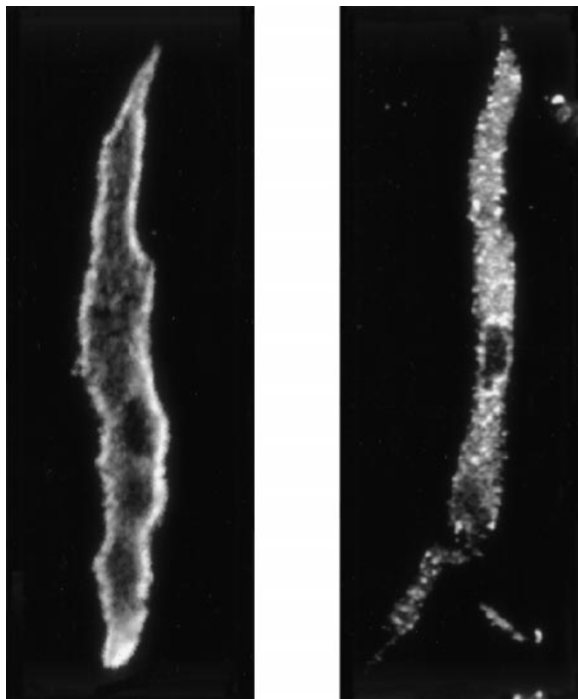

Fig. 1. Translocation of Raf-1 in response to ceramide stimulation in smooth muscle cells of the rabbit colon. Raf-1 translocated to the cell membrane (*left cell*) in response to ceramide (10^{-6} M) stimulation, as observed under confocal microscopy. Preincubation of cells with vasoactive intestinal peptide (VIP; 10^{-6} M) led to inhibition of Raf-1 translocation to the membrane, and Raf-1 is seen to be distributed throughout the cytoplasm (*right cell*).

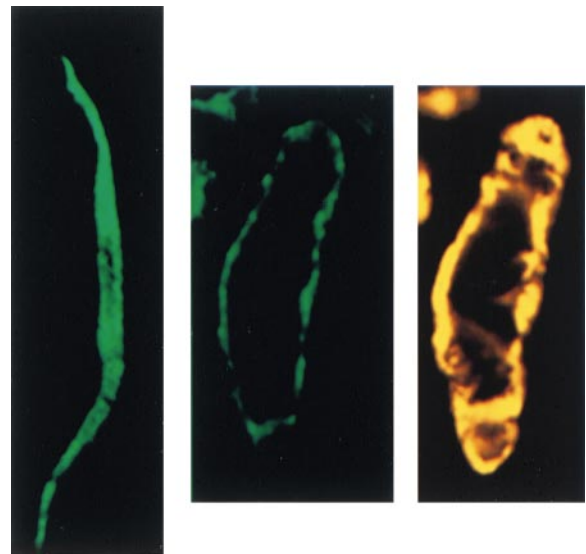

Fig. 2. Membrane translocation of protein kinase C (PKC)-α in response to ceramide stimulation and colocalization of PKC-α with mitogen-activated protein (MAP) kinase in smooth muscle cells of the rabbit colon. Confocal microscopy showing PKC-α distributed throughout the cell at rest (*left cell*). When cells were stimulated with ceramide (10^{-6} M), PKC-α translocated to the membrane (*middle cell*). PKC-α translocation and colocalization with MAP kinase in response to stimulation by ceramide (10^{-6} M) is shown in *right cell*.

Colocalization of HSP27 and Actin on Contraction of Smooth Muscle Cells

We have used the technique described above using immunofluorescent labeling of two proteins to determine the localization of HSP27 and actin in relaxed and contracted cells. Figure 4 shows HSP27 in the control state (*left cell*) and HSP27 reorganization into crossbands in response to stimulation by ceramide (10^{-6} M) (*right cell*). Actin is ubiquitous and seems to be distributed throughout the cell at rest (Fig. 5, *left cell*). Results obtained from colocalization studies of HSP27 with actin show that, after ceramide (10^{-6} M)-induced contraction, HSP27 and actin aggregate into a dense arrangement with strong colocalization in the areas of the crossbands (Fig. 5, *middle* and *right*). Colocalization of HSP27 with actin as seen under confocal microscopy would suggest a possible interaction of HSP27 with other contractile proteins involved in smooth muscle contraction, such as myosin, tropomyosin, and caldesmon.

Immunoprecipitation of HSP27 with Actin, Myosin, Tropomyosin, and Caldesmon

To elucidate possible interactions between HSP27 and contractile proteins, immunoprecipitates of HSP27 from lysates of untreated cells were subjected to SDS-PAGE, followed by sequential probing with anti-HSP27 monoclonal antibody, anti-actin monoclonal antibody, anti-myosin monoclonal antibody, anti-tropomyosin monoclonal antibody, and anti-caldesmon monoclonal antibody. All five proteins (HSP27, actin, myosin, tropomyosin, and caldesmon) were detected (Fig. 6): HSP27 ran at a molecular mass of ~31 kDa on SDS-PAGE, whereas actin could be detected at ~43 kDa. Tropomyo-

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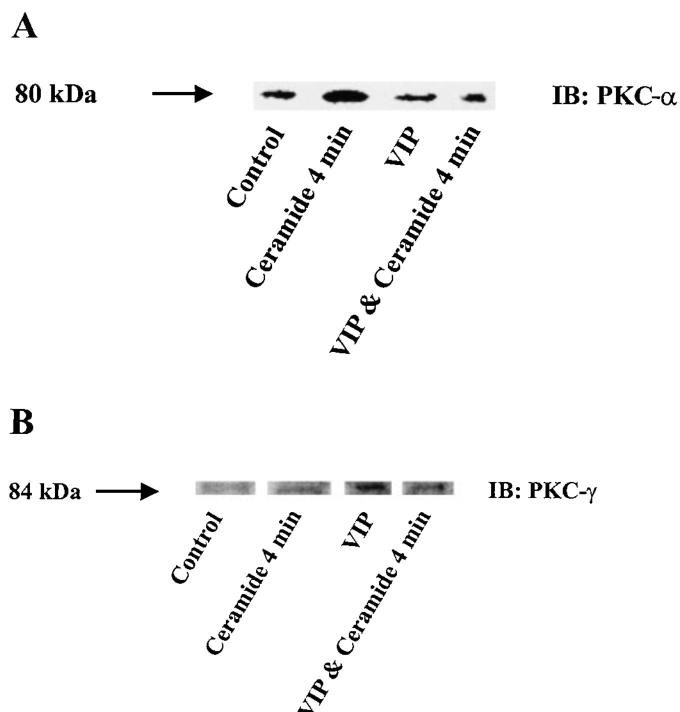

Fig. 3. Translocation of PKC-α to the particulate (membrane) fraction of rabbit colon smooth muscle cell lysate. Lysates were spun at 100,000 *g* for 30 min. Particulate fractions were subjected to SDS-PAGE and immunoblotted (IB) with the PKC-α (*A*) or PKC-γ (*B*) antibody. *A* and *B*: *lane 1*, lysate from untreated cells; *lane 2*, lysate from cells stimulated with ceramide (10^{-6} M) for 4 min; *lane 3*, lysate from cells preincubated with VIP (10^{-6} M) for 10 min; *lane 4*, lysate from cells preincubated with VIP (10^{-6} M) for 10 min and coincubated with ceramide (10^{-6} M) for 4 min. PKC-α (*A*) translocated to the membrane fraction in response to ceramide stimulation, whereas PKC-βII (result not shown) and PKC-γ (*B*) did not.

sin was seen around 36 kDa, and two myosin bands were detected around 200 and 204 kDa. Monoclonal antibody to caldesmon also detected two bands around 70 and 150 kDa. Similar results were obtained when antibody to any of the other four proteins (anti-actin monoclonal antibody, anti-myosin monoclonal antibody, anti-tropomyosin monoclonal antibody, or anti-caldesmon monoclonal antibody) was used for immunoprecipitation, followed by SDS-PAGE and probing for some or all of the other proteins in the group (data not shown). Actin, myosin, tropomyosin, and caldesmon also coimmunoprecipitated with HSP27 when cells were treated with the contractile agonist ceramide (10^{-6} M) or ACh (10^{-7} M) for 30 s or 4 min (data not shown). These results suggest that HSP27, actin, myosin, tropomyosin, and caldesmon may be interacting and may be involved in the regulation of sustained smooth muscle contraction.

Effect of ML-7 on Agonist-Induced Contraction of Colonic Smooth Muscle Cells

Freshly isolated smooth muscle cells were treated with ceramide (10^{-6} M) or ACh (10^{-7} M) for 30 s or 4 min with or without prior incubation with ML-7 (10^{-5} M), an inhibitor of MLCK. Ceramide induced smooth muscle cell contraction at 30 s (32.31 ± 3.44% decrease in cell length), which remained sustained at 4 min (35.22 ± 5.42% decrease in cell length) (Fig. 7*A*). Similarly, ACh induced contraction at 30 s (31.62 ± 1.92% decrease in cell length), which remained sustained at 4 min (34.09 ± 3.70% decrease in cell length) (Fig. 7*B*). At 30 s, 32.53 ± 1.78% of the contractile response induced by ceramide was inhibited by preincubation of the cells with ML-7 ($P < 0.05$), indicating that the remaining 67.47 ± 1.78% of the contractile effect elicited by ceramide was probably through a pathway different from myosin light chain phosphorylation (Fig. 7*A*). Similarly, at 4 min, 40.44 ± 2.56% of the sustained contractile response elicited by ceramide was inhibited by ML-7 ($P < 0.05$), indicating that 61.06 ± 4.06% of ceramide-induced contraction was probably through a different pathway from myosin light chain phosphoryla-

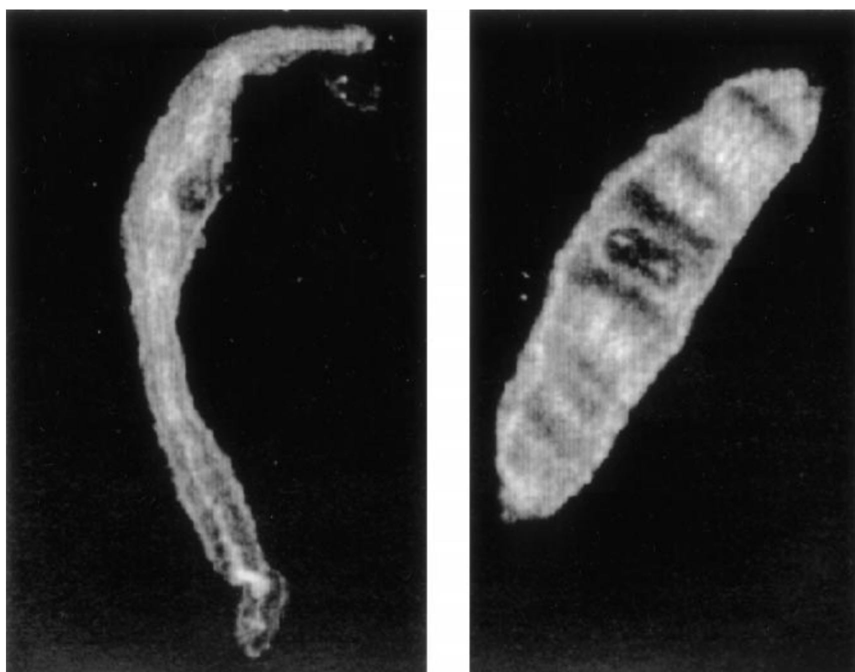

Fig. 4. Reorganization of heat-shock protein 27 (HSP27) in response to ceramide stimulation in rabbit colon smooth muscle cells. In cells preincubated with immunofluorescent antibody to HSP27 and visualized under confocal microscopy, HSP27 is distributed along the membrane and throughout the cytoplasm at rest (*left cell*). Upon contraction in response to ceramide (10^{-6} M), HSP27 reorganizes into distinct "bandlike" structures, as shown in *right cell*.

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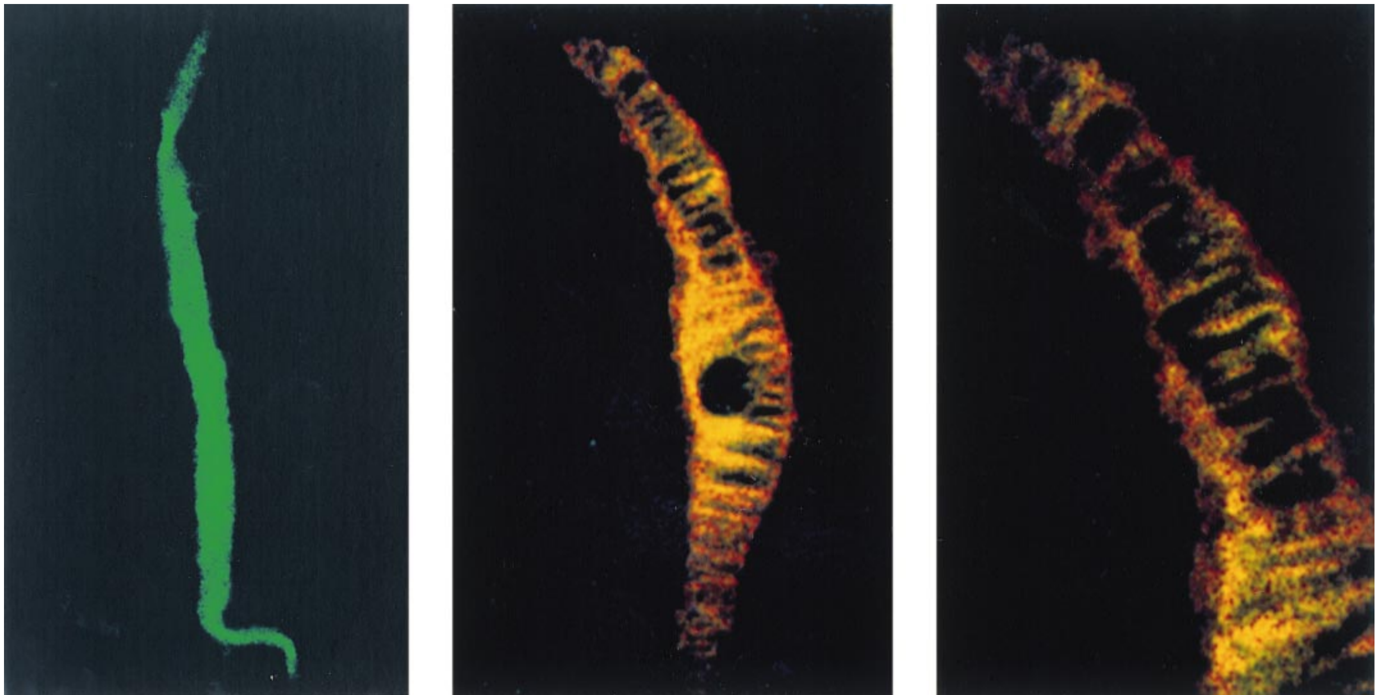

Fig. 5. Colocalization of HSP27 and actin in rabbit colon smooth muscle cells. Cells were preincubated with immunofluorescent antibody to both HSP27 and actin and visualized under confocal microscopy. Computer-generated photomicrograph of 30 pictures taken at different cross-sectional levels shows areas of colocalization as yellow [a combination of rhodamine (red) used for HSP27 and fluorescein (green) used for actin]. *Left cell* shows actin distributed throughout the cell in the resting state. When stimulated with ceramide (*right cell*, high power of same cell in *middle*), areas of colocalization (yellow) aggregate to form "bandlike" structures similar to those seen with HSP27 when subjected to ceramide stimulation (Fig. 4).

tion (Fig. 7*A*). Similar results were obtained with ACh-induced contraction; 14.82 ± 11.07% of the contractile response elicited by ACh at 30 s was inhibited by ML-7 ($P < 0.05$) (Fig. 7*B*), and 19.17 ± 12.68% of the contractile response elicited by ACh at 4 min was inhibited by ML-7 (Fig. 7*B*).

DISCUSSION

PKC seems to mediate sustained smooth muscle contraction through a cascade of signal transduction events (9, 30, 53). In this study, we have attempted to correlate some of the events that take place in the signal transduction cascade with the interaction between HSP27 and some of the contractile proteins involved in smooth muscle contraction.

Raf-1 kinase is activated by growth factors that induce its translocation from the cytoplasm to the plasma membrane through a complex mechanism, which includes its interaction with the GTP-bound form of Ras, and possibly phosphorylation of PKC and tyrosine kinases (5, 17, 39, 41). Although the exact nature of the events occurring at the membrane is still unclear, it is evident that the translocation of Raf-1 is important. Recently, Rizzo et al. (41) have shown that phosphatidic acid induces Raf-1 translocation to the membrane and also induces phosphorylation of MAP kinase. In the present study, we have shown that stimulation of colonic smooth muscle cells with ceramide leads to translocation of Raf-1 to the membrane; this effect of ceramide is inhibited by VIP, a relaxant neuropeptide. We have previously shown that bombesin- and ceramide-induced smooth muscle contraction is mediated via a signal transduction cascade that includes PKC and MAP kinase (53, 54). Further examination of this intracellular cascade revealed that stimulation of colonic smooth muscle cells with ceramide also leads to translocation of PKC-α and MAP kinase to the membrane where they colocalize. Liou and Morgan (33) have also shown agonist-induced translocation of PKC-α in cells from rat aorta. We have previously shown that ceramide induces MAP kinase phosphorylation (28, 42). Ceramide has also been found to phosphorylate HSP27 (27). Thus incubation of smooth muscle cells with the contractile agonist ceramide seems to trigger a cascade of events including Raf-1, PKC-α, and MAP kinase translocation to the plasma membrane and phosphorylation of MAP kinase and of HSP27.

Actin is ubiquitous and is distributed throughout the cell in the resting state. HSP27 is also distributed throughout the cell in the resting state. However, when stimulated with ceramide, HSP27 reorganizes inside the cell with crossband formation. When stimulated with ceramide, actin also appears to reorganize with the formation of crossbands, which do not appear to be as distinct as those formed, on reorganization, by HSP27. Both proteins appear to colocalize in the area of crossband formation. Takeuchi et al. (47) also observed redistribution of actin on activation in platelets. We further investigated the interaction of HSP27 with

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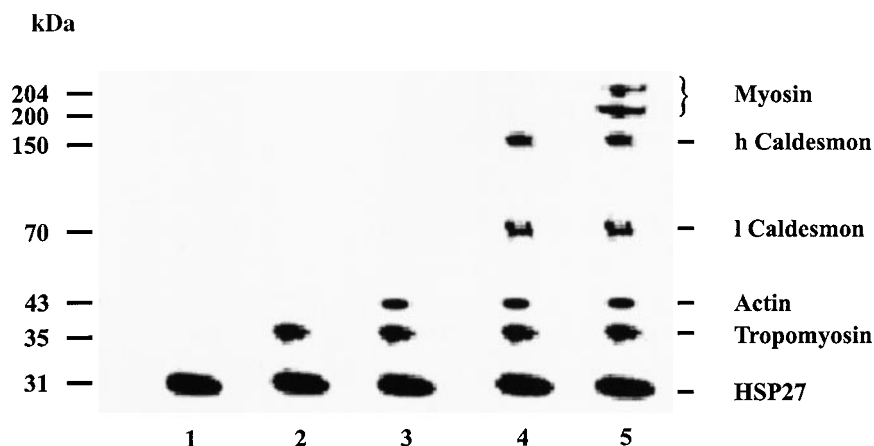

Fig. 6. Coimmunoprecipitation of actin, myosin, tropomyosin, and caldesmon with HSP27 in rabbit colon smooth muscle cells. Immunoprecipitates of HSP27 from 200 µg of protein lysate of untreated cells were subjected to SDS-PAGE and Western blotted sequentially with anti-HSP27 monoclonal antibody (*lane 1*), anti-tropomyosin monoclonal antibody (*lane 2*), anti-actin monoclonal antibody (*lane 3*), anti-caldesmon monoclonal antibody (*lane 4*), and anti-myosin monoclonal antibody (*lane 5*). All 4 proteins (actin, myosin, tropomyosin, and caldesmon) coimmunoprecipitated with HSP27: HSP27 ran at a molecular mass of ~31 kDa on SDS-PAGE, whereas actin could be detected ~43 kDa. Tropomyosin was seen around 36 kDa, and 2 myosin bands were detected around 200 and 204 kDa. Monoclonal antibody to caldesmon also detected two bands around 70 and 150 kDa. Actin, myosin, tropomyosin, and caldesmon also immunoprecipitated with HSP27 when cells were treated with the contractile agonists ceramide (10^{-6} M) or ACh (10^{-7} M) for 30 s or 4 min (data not shown). Incubation of blot in secondary antibody only was used as a negative control. Bars on *left* indicate the positions of prestained molecular mass markers. Different proteins identified are indicated on *right*.

actin and with some other contractile proteins such as myosin, tropomyosin, and caldesmon. We performed immunoprecipitation of lysates with a monoclonal antibody to HSP27 (2B4-123) (10), followed by sequential immunoblotting to probe for actin, myosin, tropomyosin, and caldesmon. At each step, it was not necessary to strip the membranes of previously bound primary and secondary antibodies because the molecular masses of all the proteins examined in the group, i.e., HSP27, actin, myosin, tropomyosin, and caldesmon, were far enough from one another to prevent overlap of protein bands. Thus the same blot containing immunoprecipitates of one of the proteins was probed consecutively for the other proteins in the group. Thus, with each additional detection, previously identified proteins could still be seen on the blot. When blots were incubated only in the secondary antibody as a negative control, the only bands that could be detected were the heavy and light chains of the IgG molecule present in the immunoprecipitate. Our results show that actin, myosin, caldesmon, and tropomyosin immunoprecipitate with HSP27. HSP27 ran at a molecular mass of ~31 kDa on SDS-PAGE, whereas actin could be detected at ~43 kDa. Tropomyosin was seen around 36 kDa, and two myosin bands were detected around 200 and 204 kDa. Monoclonal antibody to caldesmon also detected two bands around 70 and 150 kDa. These results suggest that HSP27 may be interacting with these contractile proteins and that HSP27 may play a role in the regulation of smooth muscle contraction. This observation is interesting because HSP27 appears to be the link between the signal transduction cascade and the contractile proteins, which bring about sustained smooth muscle contraction mediated by PKC. However, the mechanism by which HSP27 may interact with contractile proteins such as actin, myosin, tropomyosin, and caldesmon to regulate smooth muscle contraction is presently unclear and will require future investigations.

Actin and myosin are the major contractile proteins involved in the generation of tone by the smooth muscle. Both striated and smooth muscles contract by a "sliding filament" model, although the contractile apparatus in smooth muscle is not as highly ordered as in skeletal muscle (14). The energy required for the sliding filament mechanism to operate is provided by the hydrolysis of ATP by myosin ATPase, an enzyme activated by actin. Caldesmon, also a contractile protein, has been shown to bind not only to actin but also to myosin and calmodulin, a calcium-binding protein (13). Extracellular signal-regulated MAP kinases have been shown to phosphorylate caldesmon (2, 18). Recently, Hedges et al. (25) have also shown caldesmon to be a substrate for p38 MAP kinase. Caldesmon forms a link between actin and myosin by binding to these proteins via its COOH-terminal and NH_2-terminal ends, respectively. Caldesmon also interacts with tropomyosin, another contractile protein (13). Tropomyosin, an actin-binding protein, enhances the caldesmon-induced inhibition of the ATPase activity of myosin (13).

Several mechanisms implicated in the initiation of vascular smooth muscle contraction involve increases in intracellular calcium and lead to the activation of MLCK. MLCK phosphorylates the regulatory light chain of myosin, which activates actomyosin ATPase activity of smooth muscle (44), leading to contraction. We have carried out contraction studies in which we tested the effect of ML-7, an MLCK inhibitor, on ceramide- and ACh-induced contraction. Our results showed that only ~30–40% of the ceramide-induced

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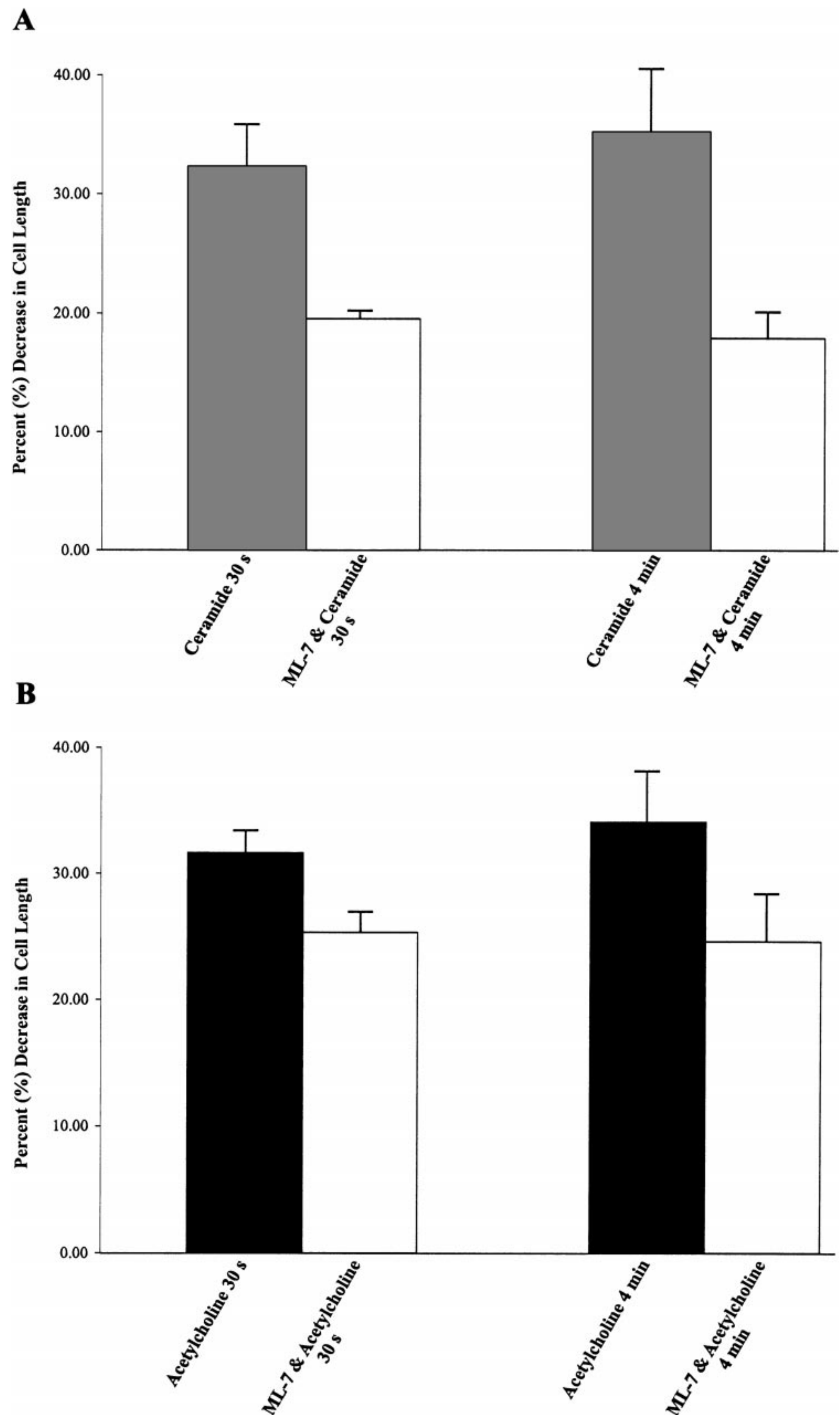

Fig. 7. Effect of 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) on agonist-induced contraction of colonic smooth muscle cells. Freshly isolated smooth muscle cells were treated with ceramide (10^{-6} M) or ACh (10^{-7} M) for 30 s or 4 min with or without prior incubation with the myosin light-chain kinase inhibitor ML-7 (10^{-5} M) as described in MATERIALS AND METHODS. *A*: ceramide-induced cell shortenings were 32.31 ± 3.44% at 30 s and 35.22 ± 5.42% at 4 min. Preincubation of cells with ML-7 resulted in a decrease in cell contraction to 19.51 ± 0.95% and 17.88 ± 1.99%, respectively. *B*: ACh-induced contractions; 31.62 ± 1.92% at 30 s and 34.09 ± 3.70% at 4 min were reduced to 25.31 ± 2.04% and 24.60 ± 4.09%, respectively, in the presence of ML-7. Data are means ± SE from 2–3 separate experiments. $P < 0.05$ by one-way ANOVA.

contraction was inhibited by ML-7 and 60–70% was not. Only ~15–20% of the contraction elicited by ACh was inhibited by ML-7. Because the total levels of contraction induced by ceramide and ACh were not completely inhibited by ML-7, it is possible that the rest of the ceramide- or ACh-induced contraction is through an alternative pathway to the one involving myosin light-chain phosphorylation. Indeed, the phos-

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phorylation-dephosphorylation of myosin has not been found to be sufficient to explain excitation-contraction of smooth muscle (30). Other regulatory pathways have therefore been implicated, at least in vascular smooth muscle contraction. Khalil and Morgan (30) have suggested that PKC may function as a second signaling pathway. Moreover, data show that vascular and gastrointestinal muscles can maintain contraction in the absence of increases in myosin light-chain phosphorylation and energy consumption (1, 22). PKC activation may contribute to the maintenance of force through a kinase cascade involving MAP kinase and increases in the phosphorylation of the actin regulatory protein caldesmon (15, 22, 24, 29, 30, 46). Thus the mechanisms by which the long-lasting contraction (referred to as the "latch state") is maintained have not been clarified.

On the basis of our current findings and our previous results, we propose the existence of an intracellular signaling cascade in colonic smooth muscle that results in the phosphorylation of HSP27 and the association of HSP27 with some contractile proteins, particularly actin, leading to sustained smooth muscle contraction. This cascade is initiated by PKC and Raf-1 translocation, both leading to MAP kinase kinase (MEK) and MAP kinase activation. VIP appears to have an inhibitory effect at several points in this cascade: VIP blocks bombesin-induced, Raf-1-mediated activation of MEK (34) and ceramide-induced PKC activation. It is noteworthy that VIP also inhibits agonist-stimulated HSP27 phosphorylation (34) as well as contraction (35). In addition, calphostin C inhibits agonist-stimulated HSP27 phosphorylation (27) and contraction. The inhibitory effects of VIP and calphostin C on both HSP27 phosphorylation and contraction would suggest that HSP27 phosphorylation might be necessary for contraction. Our data show that HSP27 colocalizes with actin, as observed under confocal microscopy, and we have also shown that both proteins coimmunoprecipitate. It is therefore possible that HSP27 association with actin is required for contraction. In addition, HSP27 interacts with other contractile proteins such as myosin, tropomyosin, and caldesmon. It is possible that actin is the major contractile protein keeping the complex together. In our study, we have found that HSP27 immunoprecipitates with caldesmon; thus it is possible that the MAP kinase signaling cascade, which leads to HSP27 phosphorylation, also causes phosphorylation of caldesmon. We therefore propose that our current and previous data confirm that HSP27 plays an important role in sustained smooth muscle contraction.

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Address for reprint requests and other correspondence: K. N. Bitar, Division of Pediatric Gastroenterology, Univ. of Michigan Medical School, 1150 W. Medical Center Dr., MSRB I, Rm. A520, Ann Arbor, MI 48109-0656 (E-mail: bitar@umich.edu).

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