Role of thin-filament regulatory proteins in relaxation of colonic smooth muscle contraction

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Somara S, Gilmont R, Bitar KN. Role of thin-filament regulatory proteins in relaxation of colonic smooth muscle contraction. Am J Physiol Gastrointest Liver Physiol 297: G958–G966, 2009. First published September 3, 2009; doi:10.1152/ajpgi.00201.2009.—Coordinated regulation of smooth muscle contraction and relaxation is required for colonic motility. Contraction is associated with phosphorylation of myosin light chain (MLC20) and interaction of actin with myosin. Thin-filament regulation of actomyosin interaction is modulated by two actin-binding regulatory proteins: tropomyosin (TM) and caldesmon (CaD). TM and CaD are known to play crucial role in actomyosin interaction promoting contraction. Contraction is associated with phosphorylation of the small heat shock protein HSP27, concomitant with the phosphorylation of TM and CaD. Phosphorylation of HSP27 is attributed as being the prime modulator of thin-filament regulation of contraction. Preincubation of colonic smooth muscle cells (CSMC) with the relaxant neurotransmitter vasoactive intestinal peptide (VIP) showed inhibition in phosphorylation of HSP27 (ser78). Attenuation of HSP27 phosphorylation can result in modulation of thin-filament-mediated regulation of contraction leading to relaxation; thus the role of thin-filament regulatory proteins in a relaxation milieu was investigated. Preincubation of CSMC with VIP exhibited a decrease in phosphorylation of TM and CaD. Furthermore, CSMC preincubated with VIP showed a reduced association of TM with HSP27 and with phospho-HSP27 (ser78) whereas there was reduced dissociation of TM from CaD and from phospho-CaD. We thus propose that, in addition to alteration in phosphorylation of MLC20, relaxation is associated with alterations in thin-filament-mediated regulation that results in termination of contraction.

heat shock protein 27; tropomyosin; caldesmon

The key mechanism that regulates the initiation of smooth muscle contraction is mediated via myosin light chain (MLC20) phosphorylation (8, 12, 21). However, an essential event of contraction is rapid cross-bridge cycling formed by actomyosin interaction, which is modulated by the thin-filament-associated proteins that regulate actomyosin adenosine triphosphatase (ATPase) activity. The major regulatory thin-filament-associated proteins are tropomyosin (TM) and caldesmon (CaD), which associate with 14 actin monomers in ratios 2:1 (13).

TMs are rodlike helical proteins that dimerize and bind to actin. The four TM genes produce many isoforms (>40) as a result of alternative exon usage. Most are found in nonmuscle cells, but there are some specific to either striated or smooth muscle. Only two isoforms appear to be specific to smooth muscle (one from the α-TM gene and the other from the β-TM gene). In smooth muscle cells, TM is an integral part of the thin filament. TM is a two-chain archetypal α-helical coiled coil whose periodic interactions with the F-actin helix are critical for the stabilization of the smooth muscle actin contractile filaments and the regulation of muscle contraction. TM associates end to end along the actin filament and cooperatively regulates its function. TM end-to-end overlap complexes, comprised of the NH2 terminus of one TM and the COOH terminus of another, facilitate the formation of continuous TM strands along the actin filament. PKC-mediated phosphorylation of TM is associated with colonic smooth muscle contraction (18). TM enhances the cooperative activation of actomyosin such that the equilibrium between on and off states is shifted to the on state.

Another regulatory actin-binding contractile protein is CaD, which along with calmodulin control the TM-mediated transition between on and off activity states. The CaD-calmodulin complex regulates the activity of the thin filament in response to calcium. In the helical construction of smooth muscle thin filament, CaD forms a continuous strand along the actin filament parallel to TM. CaD is an inhibitor of actomyosin ATPase and motility. Both actin binding and actomyosin ATPase inhibition are enhanced in the presence of TM. Thus TM and CaD behave as allosterically coupled ligands of actin. The inhibitory action of CaD is modulated by both calcium and phosphorylation. CaD is phosphorylated on ser789 during colonic smooth muscle contraction.

Another protein whose phosphorylation plays a crucial role in smooth muscle contraction is the small heat shock protein HSP27. During agonist-induced contraction, HSP27 undergoes a significant and sustained phosphorylation. HSP27 phosphorylation has an impact on the signaling pathways as well as on the thin-filament regulation of colonic smooth muscle contraction. Stimulation of freshly isolated colonic smooth muscle cells (CSMC) with contractile agonists induces coimmunoprecipitation of HSP27 with the contractile proteins actin, TM, and CaD (10). In agonist-stimulated CSMC, phosphorylation of HSP27 enhances the association of HSP27 with TM (18). CSMC transfected with non-phospho-HSP27 mutant show a decrease in the association of TM with HSP27 (18). Thus phosphorylation of HSP27 plays an important role in the regulation of smooth muscle contraction at the level of thin-filament protein interactions. We have proposed a model whereby acetylcholine (ACh) induces phosphorylation of CaD at ser789, resulting in association of ser789 phospho-CaD with phospho-HSP27 leading to an essential conformational change. This conformational change results in dissociation of phospho-CaD from TM, which leads to the sliding of TM on actin, thus exposing the myosin-binding sites on actin (17).

Contraction occurs when the myosin heads of the thick filaments interact with the actin molecules of the thin filaments. Relaxation of smooth muscle occurs either as a result of removal of the contractile stimulus, i.e., removal of ACh, or by the direct action that stimulates inhibition of the contractile...
mechanism, i.e., association or dissociation, phosphorylation or dephosphorylation of contractile proteins even in the presence of contractile stimulus. Contraction is regulated by a balance between the activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). The process of relaxation requires a decreased intracellular Ca^2+ concentration and increased MLCP activity. Most previous investigations have suggested that cyclic nucleotide-dependent relaxation occurs through inhibition or reversal of the Ca^2+/MLCK/MLC20 pathway (20). Contraction is associated with phosphorylation of CaD, TM, and HSP27 concomitant to TM association with HSP27 and dissociation from CaD. Relaxation could be the reverse of the contractile pathway, i.e., blocking the effect of contractile stimulus by the direct action that stimulates inhibition of contraction. Thus studies were carried out to investigate phosphorylation of TM, CaD, HSP27, and association of these contractile proteins with each other in relaxation milieu.

The neurotransmitter vasoactive intestinal peptide (VIP), which causes the relaxation of intact segments of gastrointestinal (GI) circular smooth muscles, was used in these studies. This investigation focused on the role of thin-filament-associated regulatory protein in relaxation. Preincubation of CSMC with VIP inhibited the phosphorylation of HSP27 in response to ACh treatment. Preincubation of CSMC with VIP also exhibited inhibition of phosphorylation of TM, CaD (ser789), and PKC-α (ser657). Furthermore, preincubation of CSMC with VIP decreased the association of TM with HSP27 whereas no change was observed in association of TM with CaD. Thus we propose that relaxation-associated inhibition of HSP27 phosphorylation leads to alterations in tridimensional remodeling of the thin-filament binding proteins, resulting in inhibition of contraction and thereby initiating relaxation. We therefore postulate that relaxation of CSMC is mediated by reduction in the phosphorylation of HSP27, which mediates regulation of thin-filament dynamics through alterations in phosphorylation of thin-filament proteins and/or in their macromolecular associations.

MATERIALS AND METHODS

Materials

The following reagents were purchased: monoclonal mouse anti-TM antibody and monoclonal mouse anti-CaD from Sigma, St. Louis, MO; rabbit polyclonal anti-phospho-HSP27 (ser78) antibody and rabbit polyclonal anti-phospho-CaD (ser789) antibody from Santa Cruz Biotechnology, Santa Cruz, CA; rabbit anti-phospho-PKC-α (ser657) antibody from Millipore, Billerica, MA; mouse monoclonal anti-HSP27 antibody from Stressgen; and secondary antibodies anti-rabbit antibody conjugated to horseradish peroxidase, anti-rabbit antibody conjugated to horseradish peroxidase from Bio-Rad Laboratories, Hercules, CA, and goat anti-mouse IgG (Fc specific) horseradish peroxidase conjugate from Millipore; polyvinylidene fluoride (PVDF) membranes from Bio-Rad Laboratories; protein G Sepharose and enhanced chemiluminescence (ECL) detection reagents from Amer sham Biosciences, Piscataway, NJ; autoradiography film (HyBlot CL) from Denville Scientific, Metuchen, NJ; G-418, penicillin-streptomycin, FBS, collagen IV, and DMEM from Gibco BRL Grand Island, NY; and collagenase type II from Worthington, Lakewood, NJ. All other reagents were purchased from Sigma.

Methods

Preparation of smooth muscle cells from the rabbit rectosigmoid. Smooth muscle cells of rabbit rectosigmoid colon were isolated as described previously (1, 2). Briefly, circular smooth muscle layer from the distal colon from New Zealand rabbits was removed by sharp dissection. A 5-cm length of the rectosigmoid, oral to the junction, was dissected and digested with collagenase to yield isolated smooth muscle cells. The tissue was incubated for two successive 1-h periods at 31°C in 15 ml HEPES (pH 7.4) (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/ml, Worthington CLS type II), 0.01 (wt/vol) soybean trypsin inhibitor, and 0.184 (wt/vol) DMEM. After the end of the second enzymatic incubation period, the medium was filtered through a Nitex mesh. The partially digested tissue left on the filter was washed four times with 10 ml of collagenase-free buffer solution. The tissue was transferred into 15 ml of fresh collagenase-free buffer solution, and the cells were gently dispersed. The harvested cells were resuspended in collagenase-free HEPES buffer (pH 7.4) and plated in DMEM with 10% FBS, antibiotics, and 0.5% l-glutamine. Cells were grown to confluence to be used for experiments.

Immunoprecipitation. Antibody (1–2 μg) was added to 500 μl of sample protein in 500 μl of lysis buffer and rocked overnight at 4°C. Fifty microliters of 50% protein G-Sepharose bead slurry was then added and the mixture was rocked at 4°C for 2 h. The beads bound with proteins were then collected by centrifugation at 14,000 g for 3 min at 4°C. The supernatant was discarded and the bead pellet was washed three times at room temperature with Tris-buffered saline (TBS) bead wash buffer (20 mM Tris·HCl, 150 mM NaCl, pH 7.6). The beads were then resuspended in 25 μl of 2 × sample buffer and boiled for 5 min. Proteins from the immunoprecipitatses were separated on SDS-PAGE and transferred to PVDF membrane. The membrane was immunoblotted with the desired antibodies as described previously (18). Replicates of experiments were performed using completely separate sets of cells.

Immunoblotting. The proteins were separated on SDS-PAGE and electrophoretically transferred to PVDF membrane as described previously (18). The membrane was blocked with 5% nonfat dry milk for 1 h and incubated in an appropriate dilution of primary antibody in 5% nonfat dry milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h. The membrane was washed thrice with TBST to remove unbound primary antibody for 15 min per wash at room temperature. The membrane was then incubated in an appropriate dilution of secondary antibody in 5% nonfat dry milk in TBST for 1 h at room temperature. The membrane was washed three times with TBST for 15 min per wash at room temperature to remove unbound secondary antibody. The membrane was then incubated with ECL reagent for 1 min. The proteins were detected on the membrane by immediately exposing the membrane to the film for 30 s and 1 min.

Data analysis. The experimental protocol calls for cells isolated from circular muscle of the colon of each animal to be divided into five groups: 1) control unstimulated cells; 2) cells stimulated with ACh for 30 s; 3) cells stimulated with ACh for 4 min; 4) cells preincubated with VIP for 20 min, stimulated with ACh for 30 s; 5) cells preincubated with VIP for 20 min, then stimulated with ACh for 4 min. In each set of experiments, the response obtained was compared with control unstimulated cells by ANOVA. The response was considered significant (P-value) at equal to or less than 0.05. The mean values are the mean of the response obtained from different set of cells isolated from different animals, which is represented by n.

Data analysis was done as described previously (18). Briefly, Western blot bands were quantitated by use of a densitometer (model GS-700, Bio-Rad Laboratories), and band volumes (absorbance units × mm^2) were calculated and expressed as a percentage of the total volume. Band data are within the linear range of detection for each antibody used. The control band intensity was standardized to
RESULTS

VIP-Induced Changes in Phosphorylation of Thin-Filament-Associated Proteins

Contraction is associated with the phosphorylation of TM, CaD, and the phosphorylation of small heat shock protein HSP27. Phosphorylation of HSP27, TM, and CaD plays a pivotal role in promoting actomyosin interaction. Investigations were done to examine the effect of VIP, a relaxant neurotransmitter, on the phosphorylation of TM, CaD, and HSP27.

VIP-induced inhibition of HSP27 phosphorylation in rabbit CSMC. Contraction of CSMC is associated with agonist-induced phosphorylation of HSP27. To examine the effect of the relaxant neurotransmitter VIP, CSMC were either directly stimulated with 0.1 μM ACh for 30 s and 4 min or preincubated with 1 μM VIP for 20 min with subsequent stimulation with 0.1 μM ACh for 30 s and 4 min. Whole cell lysates were immunoblotted with anti-phospho-HSP27 (ser78) antibody. Preincubation of CSMC with 1 μM VIP resulted in an inhibition of HSP27 phosphorylation (106.34 ± 11.97% after ACh 30 s; 105.36 ± 9.91% after ACh 4 min; n = 3, P > 0.05) compared with CSMC not incubated with VIP (175.24 ± 11.97% after ACh 30 s; 176.56 ± 11.69% after ACh 4 min; n = 3, P ≤ 0.05) (Fig. 1). VIP-induced inhibition of HSP27 phosphorylation correlated with the VIP-induced relaxation of smooth muscle. This result was consistent with earlier reports showing that VIP inhibited sustained contraction in freshly isolated smooth muscle cells (14). Inhibition of agonist-induced phosphorylation of HSP27 appeared to be a crucial event in modulating thin-filament-mediated regulation of colonic smooth muscle contraction.

VIP-induced inhibition of CaD phosphorylation in rabbit CSMC. CSMC were either directly stimulated with 0.1 μM ACh for 30 s and 4 min or preincubated with 1 μM VIP for 20 min with subsequent stimulation with 0.1 μM ACh for 30 s and 4 min. Whole cell lysates were immunoblotted with anti-phospho-CaD (ser789) antibody. Preincubation of CSMC with 1 μM VIP resulted in inhibition of phosphorylation of CaD (101.06 ± 7.24% after ACh 30 s; 108.24 ± 5.88% after ACh 4 min; n = 3, P > 0.05) compared with CSMC not incubated with VIP (170.88 ± 9.84% after ACh 30 s; 181.19 ± 4.23% after ACh 4 min; n = 3, P ≤ 0.05) (Fig. 2). CaD aids in the positioning of TM on actin to block myosin-binding sites on actin. VIP-induced inhibition of CaD phosphorylation could lead to inhibition of the displacement of TM on actin. Further investigations were carried out to examine the phosphorylation status of TM during relaxation of smooth muscle.

VIP-induced inhibition of TM phosphorylation in rabbit CSMC. TM inhibits the binding of actin with myosin by positioning itself on actin to cover myosin-binding sites on actin (4). ACh induces the phosphorylation of TM in CSMC (18), and TM phosphorylation plays a pivotal role in actomyosin interaction. We thus investigated phosphorylation status of TM during VIP-induced relaxation of CSMC. Smooth muscle cells isolated from rabbit colon were stimulated with 0.1 μM ACh for 30 s and 4 min with and without preincubation with 1 μM VIP. Whole cell lysates were immunoprecipitated with anti-phospho-ser657 antibody and immunoblotted with anti-TM antibody. Immunoblot analysis of whole cell lysates of rabbit CSMC showed very little phosphorylation of TM during relaxation of smooth muscle with 1 μM VIP. The band intensities of samples from treated cells were compared with the control and expressed as percent change from the control.

VIP-induced inhibition of PKC-α phosphorylation in rabbit CSMC. In CSMC, PKC-α is translocated to membrane and phosphorylated at ser657, leading to its activation in response to ACh stimulation. Activated PKC-α mediates the phosphorylation of HSP27, CaD, and TM. It was of interest to examine whether VIP-induced inhibition of ACh-induced phosphorylation of HSP27, CaD, and TM was due to alterations in PKC-α phosphorylation. Confluent cultured CSMC were stimulated with 0.1 μM ACh for 30 s or 4 min with and without preincubation with 1 μM VIP. Immunoblot analysis of whole cell lysate with phospho-specific anti-PKC-α antibody showed very little or no phosphorylation of PKC-α in the cells preincubated with VIP (104.57 ± 10.09% after ACh 30 s; 105.06 ± 10.19% after ACh 4 min; n = 3, P > 0.05) compared with cells not exposed to VIP (174.93 ± 9.87% after ACh 30 s; 178.99 ± 9.80% after ACh 4 min; n = 3, P ≤ 0.05) (Fig. 4). These data suggest that VIP-induced inhibition of phosphorylation of HSP27, CaD, and TM could be due to VIP-induced inhibition of PKC-α phosphorylation.
VIP-Induced Changes in Interaction of Thin-Filament-Associated Proteins

TM binds to actin whereas CaD binds with actin-TM complex in resting smooth muscle cells. These bindings block the myosin-binding sites on actin, restraining actomyosin interaction. During agonist-induced contraction, TM and CaD are phosphorylated. These phosphorylations reduce their affinity for actin while concomitantly increasing their affinity for HSP27. This leads to exposure of myosin-binding sites on actin, resulting in actomyosin interaction. Since the neurotransmitter VIP, which causes relaxation of intact segments of GI circular smooth muscles, is associated with alterations in phosphorylation of HSP27, TM, and CaD, further investigations were done to examine whether VIP affects the interaction of these proteins.

Interaction of TM with HSP27. VIP-INDUCED INHIBITION OF TM-HSP27 INTERACTION IN RABBIT CSMC. Interaction of TM with phosphorylated HSP27 aids in the sliding of the TM on actin to expose myosin-binding sites for actomyosin interaction leading to contraction (18). Thus investigations were carried out to examine the effect of VIP on the association of TM with HSP27. Confluent CSMC were stimulated with 0.1 μM ACh for 30 s or 4 min with and without preincubation with 1 μM VIP. Proteins from whole cell lysate were immunoprecipitated with anti-TM antibody and immunoblotted with anti-HSP27 antibody. No significant amount of HSP27 in TM immunoprecipitates of CSMC preincubated with VIP was observed (111.08 ± 4.84% after ACh 30 s; 113.50 ± 5.35% after ACh 4 min; n = 3, P > 0.05) compared with cells not exposed to VIP (170.93 ± 4.23% after ACh 30 s; 179.24 ± 7.18% after ACh 4 min; n = 3, P ≤ 0.05) (Fig. 5A). The data thus suggest that CSMC exhibited reduced ACh-mediated interaction of TM with HSP27 upon preincubation with VIP. This reduction in interaction could be related to possible VIP-induced inhibition of HSP27 phosphorylation and/or due to VIP-induced inhibition of TM phosphorylation. Investigations were further carried out to examine the TM interaction with phospho-HSP27 during relaxation.

VIP-INDUCED INHIBITION OF TM-PHOSPHO-HSP27 INTERACTION IN RABBIT CSMC. Furthermore, investigations were carried out to confirm VIP-mediated inhibition of ACh-induced association of TM with phospho-HSP27. Confluent CSMC were stimulated with 0.1 μM ACh for 30 s or 4 min with and without preincubation with 1 μM VIP. Proteins from whole cell lysate were immunoprecipitated with anti-TM antibody and the immunoprecipitates were separated on SDS-PAGE. Separated proteins were transferred to PVDF membrane, and the membrane was immunoblotted with anti-phospho-HSP27 (ser78) antibody. Data showed no significant increase in the association of phospho-HSP27 with TM in CSMC preincubated with VIP (101.42 ± 4.16% after ACh 30 s; 98.34 ± 4.12% after ACh 4 min; n = 3, P > 0.05) compared with cells not exposed to VIP (166.77 ± 6.45% after ACh 30 s; 179.87 ± 5.64% after ACh 4 min; n = 3, P > 0.05).
These data suggest that reduced interaction of TM with HSP27 upon preincubation with VIP could be primarily due to either VIP-induced inhibition of phosphorylation of either or both of HSP27 or TM. Interaction of TM with CaD. VIP-INDUCED INHIBITION OF DISSOCIATION OF TM-CAD IN RABBIT CSMC. TM is known to be associated with CaD and inhibit actomyosin ATPase activity. Upon contractile stimulation CaD gets phosphorylated and dissociates from TM, TM then slides on actin to expose myosin-binding sites for actomyosin interaction leading to contraction. Thus investigations were carried out to examine the effect of VIP on the association of TM with CaD. Confluent CSMC were stimulated with 0.1 μM ACh for 30 s or 4 min with and without preincubation with 1 μM VIP. Proteins from whole cell lysates were separated on SDS-PAGE and were transferred onto PVDF membrane. The membrane was then immunoblotted with anti-phospho-PKC-α (ser657) antibody. Inset shows the Western blot representing the data. Graph representing the data shows that preincubation of CSMC with VIP shows a significant inhibition of PKC-α phosphorylation compared with CSMC not exposed to VIP. This confirms that the reduced interaction between TM and HSP27 is due to the reduced amount of phospho-HSP27 available for association.
whole cell lysate were immunoprecipitated with anti-TM antibody and immunoblotted with anti-CaD antibody. CSMC preincubated with 1 μM VIP exhibited no difference in the amount of CaD in TM immunoprecipitates (99 ± 4.26% after ACh 30 s; 97.26 ± 1.19% after ACh 4 min; n = 3, P > 0.05) compared with reduced association of TM with CaD exhibited by CSMC not exposed to VIP (69.29 ± 1.86% after ACh 30 s; 60.30 ± 3.90% after ACh 4 min; n = 3, P ≤ 0.05) (Fig. 6A). These data indicate that pretreatment with VIP inhibits the ACh-dependent dissociation of TM from CaD required for smooth muscle contraction. ACh-induced phosphorylation of both CaD and TM weakens the association of CaD with TM. Since VIP induces inhibition of phosphorylation of both CaD and TM, there may be no alteration in the association of CaD with TM. Since phosphorylation of CaD was inhibited by VIP, further investigations were done to examine the association of phospho-CaD with TM.

**VIP-INDUCED INHIBITION OF DISSOCIATION OF TM-PHOSPHO-CaD IN RABBIT CSMC.** Since preincubation with VIP inhibited phosphorylation of CaD along with inhibition of dissociation of TM with CaD, investigations were done to confirm whether the dissociation of TM with CaD was inhibited owing to VIP-induced inhibition of phosphorylation of CaD. Confluent CSMC were stimulated with 0.1 μM ACh for 30 s or 4 min with and without preincubation with 1 μM VIP. Proteins from whole cell lysate were immunoprecipitated with anti-TM antibody and immunoblotted with anti-phospho-CaD (ser789) antibody. The amount of phospho-CaD in TM immunoprecipitates of CSMC preincubated with 1 μM VIP remained same as control (101.45 ± 3.32% after ACh 30 s; 96.57 ± 2.61% after ACh 4 min; n = 3, P > 0.05) compared with cells not exposed to VIP, which showed a decreased amount of phospho-CaD in TM immunoprecipitates, i.e., dissociation of TM from CaD (71.95 ± 3.03% after ACh 30 s; 66.62 ± 4.75% after ACh 4 min; n = 3, P ≤ 0.05) (Fig. 6B). The data thus suggest that CSMC exhibited reduced dissociation of TM with phospho-CaD upon preincubation with VIP. This reduction in interaction could be related to possible VIP-induced inhibition of CaD phosphorylation and/or due to VIP-induced inhibition of TM phosphorylation.

**DISCUSSION**

Phosphorylation of the 20-kDa myosin light chain is required to initiate contraction and rapid cross-bridge cycling in smooth muscles (21). Cross bridges are formed by the interaction of myosin S1 heads with actin molecules (23). However, research suggests a role for the thin-filament binding proteins CaD and TM (3, 4, 7, 9, 16, 18, 22) in cross bridge formation that regulate contraction at thin-filament level. TM forms a dimer by end-to-end interaction and wraps around the actin molecule to stabilize the thin-filament assembly (25). TM averts the activation of myosin Mg$^{2+}$-ATPase owing to its inhibitory position on actin, by covering myosin-binding sites (4). CaD is an actin-TM binding protein that spans 2 TM and 14 actin monomers such that the COOH-terminal region of CaD interacts with actin and TM, whereas the NH$_2$-terminal domain of CaD may interact with the adjacent molecule of TM (24). CaD also regulates smooth muscle contraction by inhibiting activation of myosin Mg$^{2+}$-ATPase. CaD-TM interaction on actin seems to play a crucial role in modulating actin-

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**Fig. 6.** A: VIP-induced inhibition of dissociation of TM from CaD in rabbit CSMC. Confluent CSMC were stimulated with 0.1 μM ACh for 30 s or 4 min with and without preincubation with 1 μM VIP. Proteins from whole cell lysate were immunoprecipitated with anti-TM antibody and the immunoprecipitates were separated on SDS-PAGE. Separated proteins were transferred to PVDF membrane and the membrane was immunoblotted with anti-CaD antibody. Inset shows the Western blot representing the data. Graph representing the data shows that CSMC preincubated with VIP exhibited no difference in the amount of CaD in TM immunoprecipitates compared with reduced association of TM with CaD exhibited by CSMC not exposed to VIP. **B:** VIP-induced inhibition of dissociation of TM from phospho-CaD in rabbit CSMC. Confluent CSMC were stimulated with 0.1 μM ACh for 30 s or 4 min with and without preincubation with 1 μM VIP. Proteins from whole cell lysate were immunoprecipitated with anti-TM antibody and the immunoprecipitates were separated on SDS-PAGE. Separated proteins were transferred to PVDF membrane and the membrane was immunoblotted with anti-phospho-CaD (ser789) antibody. Inset shows the Western blot representing the data. Graph representing the data shows that CSMC preincubated with VIP exhibited no difference in the amount of CaD in TM immunoprecipitates compared with reduced association of TM with phospho-CaD exhibited by CSMC not exposed to VIP. This confirms that the reduced dissociation of TM from CaD is due to the reduction in phosphorylation of CaD.
myosin interaction. It has been suggested that CaD phosphorylation and/or the binding of CaD with calcium-calmodulin complex cooperate in modulating the binding of CaD to actin-TM, leading to actomyosin interaction and activation of actomyosin ATPase (9) Thus CaD phosphorylation leads to its dissociation from actin-TM complex and regulates smooth muscle contraction by weakening TM affinity for actin (9, 16).

Relaxation, i.e., inhibition or termination of contraction, has been known to involve increased myosin phosphatase activity with a concomitant decrease in the intracellular calcium levels (11). However, very little is known about the regulation of actin and myosin dynamics at thin-filament level during relaxation. We investigated the modulation of thin-filament regulatory proteins in a relaxation milieu. Investigations were carried out to study the phosphorylation and association of thin-filament binding proteins following preincubation of smooth muscle cells with VIP. The neurotransmitter VIP, which causes relaxation of intact segments of GI circular smooth muscle, induced the inhibition of ACh-induced phosphorylation of HSP27, CaD, and TM. Pretreatment with VIP inhibited the ACh-induced association of TM with HSP27 and inhibited the dissociation of TM from CaD in smooth muscle cultured cells from rabbit colon.

Relevance of Phosphorylation of CaD and TM

Studies have shown that contraction is associated with phosphorylation of HSP27, CaD, and TM. These proteins modulate the cross bridging of actin and myosin. These studies show that upon relaxation of CSMC with VIP there is an inhibition of contraction-associated phosphorylation of HSP27, of TM, and of CaD. Alteration in phosphorylation of HSP27 may affect its functional role in contraction resulting in inhibition or termination of contraction leading to relaxation. Alteration in phosphorylation of CaD and TM may lead to prevention of the interaction of actin with myosin, which further augments the termination of contraction leading to relaxation. Relaxation of CSMC with VIP exhibited inhibition of ACh-induced phosphorylation of PKC-α. Since PKC-α phosphorylates both TM and CaD, inhibited phosphorylation of PKC-α could be the reason for inhibition of phosphorylation of TM and CaD. Attenuation of PKC-α phosphorylation can again be linked to decreased HSP27 phosphorylation since phosphorylated HSP27 has been reported to be an essential component for translocation and activation of PKC-α (15). Previous studies have shown that VIP induces PKA-mediated rapid and sustained phosphorylation of HSP20 that is associated with relaxation (6). The present study provides additional mechanism by which VIP would modulate the thin-filament regulation leading to relaxation. However, further studies defining the mechanism by which VIP attenuated the phosphorylation of PKC need to be done.

Relevance of Physiological Interaction of CaD and TM

Association of different contractile proteins aid in formation of actin-myosin cross bridge. TM binds to actin and CaD binds to actin-TM in such a way that myosin-binding sites on actin are masked, leading to inhibition of Mg-ATPase activity. Phosphorylation of TM and of CaD results in their dissociation during contraction. Binding of phospho-HSP27 to phospho-CaD and to TM further stabilizes the dissociation of CaD from TM. These events result in displacement of TM on actin exposing the myosin-binding sites on actin, leading to actin-myosin interaction (16, 18). These studies show that VIP-induced relaxation of CSMC correlates with VIP-induced inhibition of ACh-stimulated association of TM with HSP27 and inhibition of ACh-stimulated dissociation of TM from CaD. These events maintain blockage of the myosin-binding sites on actin, by sustaining the position of TM on actin preventing the interaction of actin with myosin and thus leading to relaxation.

VIP-induced diminished dissociation of TM from CaD due to inhibition of phosphorylation of CaD, TM, and HSP27 in CSMC suggests that an equilibrium of phosphorylation and dephosphorylation of contractile proteins is an essential for maintenance of actomyosin interaction and thus for the maintenance of force. Relaxation is associated with dephosphorylation of HSP27, which alters the associations of thin-filament regulatory proteins and may lead to termination of contraction resulting in relaxation. So relaxation may be due to the inhibition of contraction, which allows regulatory proteins to maintain their inhibitory positions by attenuation of their phosphorylations and associations.

Relevance of Phosphorylation of PKC-α

It is interesting to note that both CaD (16) and TM (19) are phosphorylated by PKC and both bind to HSP27 in response to ACh stimulation. Although both molecules bind to actin and inhibit Mg-ATPase activity, whether their phosphorylations occur synchronously or independently is unclear. VIP-induced relaxation-associated inhibition of HSP27 phosphorylation may affect PKC-α phosphorylation since it is known that HSP27-mediated translocation of PKC-α is crucial for agonist-induced smooth muscle contraction (15) and that translocation of PKC-α is essential for its phosphorylation and activation. Thus VIP-induced inhibition of phosphorylation of PKC-α in CSMC could also be the reason for diminished phosphorylation of TM and CaD since phosphorylation of TM and CaD are PKC-mediated. VIP-induced inhibition of the phosphorylation of CaD and TM is consistent with data showing that VIP maintains the inhibition of TM with CaD. Phosphorylation of HSP27 is known to be crucial for translocation of PKC-α to the membrane for PKC-α activation (phosphorylation). VIP-mediated inhibition of HSP27 phosphorylation could thus inhibit PKC translocation and thus its activation (phosphorylation). This will lead to inhibition of PKC-mediated phosphorylation of TM and CaD. Activation of PKC-α is one of the earliest events in agonist-induced contraction, but it is also crucial for the maintenance of contraction as it regulates the inhibition of MLCP by phosphorylating CPI-17. PKC thus plays a critical role in maintaining agonist-induced contraction at two levels: thin-filament level (by phosphorylation of TM and of CaD) and thick-filament level by regulating myosin light chain phosphorylation by modulating MLCP activity. Thus, although our studies show that VIP-induced inhibition of phosphorylation of PKC-α in CSMC may be regulating the thin-filament component of smooth muscle motility, it is also possible that there is simultaneous regulation of MLCP at thick-filament level. Overall, these data suggest that phosphorylation of PKC-α may have a vital role at the thin-filament level in regulating VIP-induced relaxation.
Phosphorylation of Small Heat Shock Proteins: Molecular Switch

Phosphorylation of HSP20 has been suggested to represent a point in which the cyclic nucleotide signaling pathways converge to prevent contraction or cause relaxation (26). Phosphorylation of HSP20 has been reported to be inhibited upon phosphorylation of HSP27 (5). Contraction-associated phosphorylation of HSP27 and relaxation-associated phosphorylation of HSP20 seems to be the toggle key that defines two states of motility (contraction and relaxation). Thus we hypothesize that phosphorylation and dephosphorylation of HSP20 or HSP27 would act as a molecular switch between contraction and relaxation events. It is possible that the PKC activation (associated with HSP27 phosphorylation) or PKA/PKG activation (mediates HSP20 phosphorylation) is the regulator of this molecular switches. Further investigations studying the effect of HSP20 phosphorylations on contractile events may confirm the hypothesis.

In summary, we conclude that VIP-induced relaxation is mediated by dephosphorylation of HSP27, TM, and CaD and may proceed through the modulation of interaction of thin-filament binding proteins. Specifically, contractile functionality of TM, CaD, and HSP27 is inhibited by VIP. This results in attenuation of the interaction of actin with myosin. TM and CaD, both actin-binding proteins, are crucial for inhibition of actin-myosin interaction and of myosin ATPase activity. Contraction is associated with phosphorylation of TM and CaD. Phosphorylation of CaD displaces CaD away from TM followed by the dislocation of TM on actin whereby myosin-binding sites are exposed for binding of myosin to actin resulting in contraction. In the relaxation scenario, inhibition of TM and CaD phosphorylation maintains their association with actin, and myosin-binding sites on actin are covered by TM held in position by CaD. Therefore there is an inhibition of interaction of actin with myosin and thus inhibition or termination of contraction leading to relaxation. Whether the dephosphorylation of CaD and/or TM and/or HSP27 marks the

Fig. 7. Modulation of contraction at the thin-filament level. In CSMC, in response to ACh PKC is translocated to the membrane and activated by autophosphorylation at ser657. Activated PKC mediates the phosphorylation of the thin-filament binding protein CaD. Phosphorylation of CaD results in conformational changes and reduced affinity for TM. TM is phosphorylated by activated PKC. Phosphorylated TM slides off the actin, exposing the myosin-binding sites. Myosin binds to actin, forming actomyosin cross bridges, resulting in ATPase activation and contraction. In the presence of VIP, PKC phosphorylation is inhibited. This further inhibits downstream phosphorylation of the thin-filament binding proteins CaD and TM, reducing the interaction of actin with myosin. At resting state, TM blocks the myosin-binding sites on actin whereas CaD binds to actin-TM, maintaining TM in its position (A). Following contractile stimulation, CaD, TM, HSP27, and myosin are phosphorylated. Phosphorylated CaD changes conformation and has reduced affinity to actin-TM. Phosphorylated TM now slides on actin, exposing the myosin-binding sites on actin. Myosin binds to actin, forming actin-myosin cross bridges. Phosphorylated HSP27 binds to phosphorylated CaD and phosphorylated TM and maintains them in their positions (B). Upon preincubation with VIP, phosphorylation of CaD, TM, and HSP27 is inhibited. This results in maintaining the resting state (A).
end of the contraction cycle or the initiation of the relaxation cycle is not clear and needs further investigation. Whether phosphorylation and dephosphorylation of HSP20 and HSP27 mark the fine point between contraction and relaxation needs further investigation.

We propose a model of thin-filament regulation of CSMC relaxation (Fig. 7) whereby dephosphorylation of HSP27 modulates the dissociation of CaD from TM, leading to inhibition of actomyosin interaction, resulting in relaxation of CSMC. During the relaxed state, CaD remains bound to TM-actin, blocking the myosin-binding sites on actin. The net outcome is alteration in 3-dimensional modeling that results in prevention of actin-myosin interaction leading to relaxation of CSMC.

GRANTS
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REFERENCES