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The small heat shock-related protein, HSP20, is a cAMP-dependent protein kinase substrate that is involved in airway smooth muscle relaxation

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Abstract

Activation of the cAMP/cAMP-dependent PKA pathway leads to relaxation of airway smooth muscle (ASM). The purpose of this study was to examine the role of the small heat shock-related protein HSP20 in mediating PKA-dependent ASM relaxation. Human ASM cells were engineered to constitutively express a green fluorescent protein-PKA inhibitory fusion protein (PKI-GFP) or GFP alone. Activation of the cAMP-dependent signaling pathways by isoproterenol (ISO) or forskolin led to increases in the phosphorylation of HSP20 in GFP but not PKI-GFP cells. Forskolin treatment in GFP but not PKI-GFP cells led to a loss of central actin stress fibers and decreases in the number of focal adhesion complexes. This loss of stress fibers was associated with dephosphorylation of the actin-depolymerizing protein cofilin in GFP but not PKI-GFP cells. To confirm that phosphorylated HSP20 plays a role in PKA-induced ASM relaxation, intact strips of bovine ASM were precontracted with serotonin followed by ISO. Activation of the PKA pathway led to relaxation of bovine ASM, which was associated with phosphorylation of HSP20 and dephosphorylation of cofilin. Finally, treatment with phosphopeptide mimetics of HSP20 possessing a protein transduction domain partially relaxed precontracted bovine ASM strips. In summary, ISO-induced phosphorylation of HSP20 or synthetic phosphopeptide analogs of HSP20 decreases phosphorylation of cofilin and disrupts actin in ASM, suggesting that one possible mechanism by which HSP20 mediates ASM relaxation is via regulation of actin filament dynamics.

Keywords

asthma; actin; protein transduction; cofilin

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β -adrenergic receptor agonists (β -agonists) are widely used for the treatment of bronchospasm associated with acute asthma attacks; however, their molecular mechanisms of action are still not completely defined. β -Agonists stimulate the heterotrimeric G protein G_s , which, in turn, activates adenylyl cyclase, catalyzing the hydrolysis of ATP into cAMP (42). In airway smooth muscle (ASM), elevated cytosolic cAMP levels activate PKA, which phosphorylates several membrane and/or intracellular proteins promoting ASM relaxation and bronchodilation of contracted airways (55). The proposed mechanisms of PKA-induced relaxation include reduction of intracellular Ca^{2+} levels as well as a reduction of the sensitivity of the contractile apparatus to intracellular calcium (31). Ca^{2+} desensitization or Ca^{2+} -independent smooth muscle relaxation refers to a decrease in muscle tension at a constant Ca^{2+} concentration. PKA-mediated phosphorylation of myosin light chain kinase has been attributed to Ca^{2+} desensitization of the contractile unit (1). It has been proposed that the mechanism of Ca^{2+} desensitization is via activation of myosin light chain phosphatase (12,30,48,50,52). However, tonic phase of smooth muscle contraction can occur in the setting where intracellular Ca^{2+} levels and myosin light chain phosphorylation are at or near basal levels (14,19,32,47). In addition, cyclic nucleotide-dependent smooth muscle relaxation has been shown to be independent of myosin light chain phosphorylation (3,29,32,43,57). Collectively, these data support the possibility that Ca^{2+} -independent smooth muscle relaxation can occur through mechanisms that are independent of myosin light chain phosphorylation.

Numerous investigations have implicated the cAMP-PKA pathway in regulating the actin cytoskeleton in cultured cells (15,24,39,45). Activation of the PKA pathway has been shown to lead to morphological changes (stellation) that are associated with loss of actin stress fibers. Treatment of cultured rat aortic smooth muscle cells with dibutyryl cAMP leads to rapid, extensive, and reversible changes in their morphology including stellation and disintegration of actin filaments (10). cAMP has been demonstrated to modulate morphology of vascular smooth muscle cells by inhibiting a Rac-dependent signaling pathway (41). Inhibition of PKA led to increased F-actin content and organization into stress fibers in lung endothelial cells (40). Since tonic contraction of smooth muscle requires an intact actin cytoskeleton (thin filaments), disruption of the actin cytoskeleton is an alternate possible mechanism of smooth muscle relaxation.

A recently identified substrate protein of PKA is the small heat shock-related protein (HSP20) (4,43). PKA activation leads to increases in the phosphorylation of HSP20 on serine 16 (Ref. 4). HSP20 is highly and constitutively expressed in muscle tissues and shares considerable homology with the family of small heat shock proteins, such as HSP27, myotonic dystrophy kinase binding protein, and the crystallins (28). Agonists that stimulate HSP20 phosphorylation, as well as analogs of phosphorylated HSP20 that contain a transduction domain (allowing for cellular penetration without altering membrane permeability) and the phosphorylated serine 16, induce relaxation of smooth muscle from a variety of different species and types of smooth muscles (4,17,18,43,53,56). The mechanism by which HSP20 mediates relaxation is still not well-understood, but several reports have suggested it involves actin cytoskeletal regulation (7,43,54,56). Mesangial cells transfected with wild-type HSP20 had few stress fibers that were localized to the periphery of the cell, whereas mesangial cells transfected with a (phosphorylation-insensitive) Ser16→Ala16 mutant HSP20 had abundant stress fibers throughout the cells that did not change with activation of cAMP pathways (56). Treatment with transducible phospho-HSP20 peptide analogs containing the phosphorylation site and a protein transduction domain led to disruption of actin stress fibers and increases in G-actin in NIH/3T3 cells (16).

Cofilin is an actin-depolymerizing protein that, when phosphorylated, binds to the intracellular scaffolding protein, 14-3-3 (Refs. 5,21). When displaced from 14-3-3, cofilin becomes dephosphorylated and activated as an actin-depolymerizing protein (20,38). HSP20 also

contains a binding motif for 14-3-3. Using size-exclusion chromatography and chemical cross-linking, Chernik et al. (11) reported direct interaction between human 14-3-3 γ and intact phosphorylated HSP20 but not unphosphorylated or mutated (S16D) HSP20 in vitro. Coimmunoprecipitation studies have also shown that 14-3-3 is associated with phospho-cofilin in Swiss 3T3 cells (16). Phospho-cofilin can be displaced from 14-3-3 by spiking the immunoprecipitates with phosphopeptide analogs of HSP20. Hence, one putative mechanism by which phosphorylated HSP20 leads to alterations in cell morphology is via competing with phospho-cofilin for binding to 14-3-3. Taken together, these data suggest that activation of cyclic nucleotide signaling pathways leading to increased phospho-HSP20 may be modulating actin filament dynamics.

To investigate the potential role of HSP20 in PKA-induced ASM relaxation, stable lines of human ASM (HASM) cells expressing green fluorescent protein (GFP) or a GFP chimera of the PKA inhibitory peptide PKI (PKI-GFP; Ref. 22) were generated. These cells were used to determine morphological and biochemical changes in response to PKA stimulation. In addition, intact bovine ASM was used to further elucidate the role of PKA in β -agonist-induced bovine ASM relaxation. Given the established effect of activation of PKA on cytoskeletal dynamics (loss of actin stress fibers), we hypothesized that one of the possible mechanisms of Ca^{2+} -independent smooth muscle relaxation is via a thin filament (actin) regulatory mechanism involving PKA-dependent phosphorylation of HSP20.

Materials and Methods

Materials

Cell culture reagents including Ham's F-12 media, L-glutamine, BSA, G418, PBS, and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). DTT, HEPES, Trizma base, Triton X-100, formaldehyde, Tween-20, CaCl_2 , sodium nitroprusside, forskolin (FSK), and isoproterenol (ISO) were purchased from Sigma Chemical (St. Louis, MO). Precast acrylamide gels, SDS, Tris-glycine-SDS buffer (TGS), Tris-glycine (TG), and prestained Precision Plus Protein All Blue Standards were purchased from Bio-Rad (Hercules, CA). Phospho-HSP20 peptides (YARAAAR-QARAWLRRAP_SAPLPGLK-COOH) and HSP20 control peptides where the phosphoserine is replaced with alanine (YARAAAR-QARAWLRRAAAPLPGLK-COOH) were synthesized by UCB Bio-products-Lonza (Cambridge, MA) and American Peptide (Sunnyvale, CA). Mouse anti-HSP20 antibodies were from Advanced Immunochemical (Long Beach, CA); rabbit anti-phospho Ser16-HSP20 antibodies were generated against the phosphopeptide WLRRAP_SAP-LPGLK (17); mouse anti-vasodilator-stimulated phosphoprotein (VASP) antibodies were from BD Biosciences (San Jose, CA); rabbit anti-PKG antibody was purchased from Stressgen (Victoria, British Columbia, Canada); rabbit anti-actin were from Sigma Aldrich (St. Louis, MO); anti-GAPDH antibodies were obtained from Abcam (Cambridge, MA); rabbit anti-phospho-cofilin-2 (Ser3) antibodies were from Upstate Biotechnology (Charlottesville, VA); and rabbit anti-cofilin-2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and agonist treatment

HASM lines stably expressing GFP or PKI-GFP were generated by retroviral infection at Wake Forest University and maintained as described previously (22). HASM cultures were grown in Ham's F-12 media (Invitrogen) supplemented with 10% FBS, 25 mM HEPES, 12 mM NaOH, 1.7 mM CaCl_2 , 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 300 $\mu\text{g}/\text{ml}$ G418 (complete media). Cultures were used for 4–5 passages after transfection, and phenotype changes were monitored by the expression of PKG (6). Before treatment with agonists, cells were serum-starved for 24 h in complete media lacking FBS and containing 0.1% BSA. Serum-starved cells plated in 100-mm dishes were stimulated with vehicle (control), 10 μM FSK, or

1 μ M ISO for 10 min. Cells were rinsed once with PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4 , pH 7.4), harvested by scraping, snap-frozen in liquid nitrogen, and stored at -80°C . For stress fiber experiments, cells were plated onto 18-mm cover glasses in 60-mm dishes 1 day before experiment so that they were 65–75% confluent before agonist treatment. Serum-starved cells were treated with agonists, 50 μ M phospho-HSP20 peptides or 50 μ M HSP20 control peptides (18) as indicated, washed three times in PBS, and fixed for 30 min in 4% (wt/vol) formaldehyde prepared in PBS. Following fixation, cells were washed three times in PBS and then permeabilized in 0.1% (vol/vol) Triton X-100 in PBS for 15 min at room temperature (RT) with gentle rocking and then washed three times in PBS.

Immunofluorescence microscopy and immunoblotting

Formaldehyde-fixed and detergent-permeabilized cells were processed for indirect immunofluorescence microscopy by adding Alexa 568-conjugated phalloidin (1:1,000 in PBS; Invitrogen) and 4,6-diamidino-2-phenylindole (DAPI; 1:1,000) and letting cells gently rock in the dark at RT for 1 h. Following three washes with PBS, labeled cells were viewed using a Zeiss Axiovert 200M epifluorescence microscope (Carl Zeiss, Thornwood, NY), equipped with a Xcite light source, and GFP autofluorescence and Alexa 568-conjugated phalloidin fluorescence images were obtained using filter sets for exciting GFP (excitation 492/18 nm) and Alexa 568 (excitation 572/23 nm) and an AxioCam HR digital camera. Images were acquired and processed using AxioVision 4.5 and Photoshop 7.0 software packages, respectively.

For immunoblot analysis, control and agonist-treated cells were scraped from a 100-mm culture dish into 100- μ l UDC buffer (8 M urea, 10 mM DTT, 4% CHAPS), and the samples were rotated for 2 h at RT. Samples were clarified by centrifugation at $\sim 8,000 g$ for 10 min. Protein concentrations were determined using the Bradford assay (Pierce Chemical, Rockford, IL). Unless indicated otherwise, 20 μ g of protein was separated on 4–20% polyacrylamide mini-gels in $1\times$ TGS buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% wt/vol SDS) at 120 V for 1.5 h. Electrophoretic transfer of proteins from the gels onto polyvinylidene difluoride membranes was carried out in $1\times$ TG buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine) at 50 V for 12 h at 4°C . The blot was subsequently incubated with one of the following primary antibodies: mouse anti-HSP20 (1:4,000 dilution); rabbit anti-phospho Ser16-HSP20 (1:500); mouse anti-VASP (1:2,000); rabbit anti-PKG (1:1,000); rabbit anti-actin (1:500); anti-GAPDH (1:300); rabbit anti-phospho-cofilin (1:2,000); rabbit anti-cofilin (1:1,000); and either of one corresponding secondary antibodies, Alexa Fluor 680-conjugated affinity-purified goat anti-mouse secondary antibody (Invitrogen) or IRDye800-conjugated affinity-purified goat anti-rabbit secondary antibody (Rockland Scientific, Gilbertsville, PA). Membranes were scanned, and the intensities of selected bands were directly quantified by the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) as described previously (22).

Focal adhesion measurement

HASM cells were seeded and cultured overnight with transfer to no serum medium (culture medium supplemented with 1 mg/ml BSA) overnight before experimentation. Cells were either untreated or treated with 100 nM Hep I (thrombospondin peptides, positive control), 10 μ M FSK, 25 μ M phospho-HSP20 peptides, or 25 μ M HSP20 control peptides as described above. Cells were fixed and examined (at least 250 cells per condition) for the presence or absence of focal adhesions by interference reflection microscopy. The percentage of cells positive for focal adhesions was determined in a minimum of three independent experiments. A cell was scored positive for focal adhesions if it contained at least five focal adhesions.

Procurement of bovine ASM tissue

Fresh bovine lung was obtained from a local abattoir (Miller's Southwest Processing, Queen Creek, AZ). The tissue was immediately placed in HEPES buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 4.7 mM KCl; 1.0 mM MgSO₄; 1.0 mM NaH₂PO₄; 1.5 mM CaCl₂; and 10 mM glucose) and stored on ice during transfer to the laboratory. Briefly, a secondary airway passage was dissected from bovine lung and cut open longitudinally. The airway was then pinned at each corner to a dissection tray. Epithelium was removed from the smooth muscle tissue by gentle rubbing with a cotton-tipped applicator. A 1-cm wide by 3-cm long strip of ASM (tangential to airflow) was then carefully dissected from the structure of the airway passage. Cross-sectional strips (1.5–2 mm wide) were then cut for use in the muscle bath.

Physiological measurements

Bovine ASM strips were suspended in a muscle bath containing a bicarbonate buffer (120 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO₄, 1.0 mM NaH₂PO₄, 10 mM glucose, 1.5 mM CaCl₂, and 25 mM Na₂HCO₃; pH 7.4) and equilibrated with 95% O₂-5% CO₂ at 37°C. Force measurements were obtained with a Kent Scientific (Litchfield, CT) force transducer (TRN001) interfaced with PowerLab from AD Instruments (Colorado Springs, CO). Data were recorded with Chart software 5.1.1 (AD Instruments). Each strip was washed every 15 min with 37°C bicarbonate buffer for the first hour of equilibration, and the length was progressively adjusted until maximal tension was obtained. The tissue was allowed to equilibrate for an additional 2 h without disturbance before experimentation was initiated. Each strip was initially tested for viability with high extracellular potassium (110 mM KCl with equimolar replacement of NaCl in bicarbonate buffer), the maximal tension obtained was taken as 100%, and tension obtained with agents (agonists, antagonists) was calculated. At the end of the experiments, all strips were washed and contracted with KCl to ensure viability of the tissues.

To determine the role of endogenous HSP20 in bovine ASM relaxation, simultaneous physiological water bath experiments were conducted using identical strips of ASM dissected from the same tissue. These strips were placed in vials of bicarbonate buffer and equilibrated in a physiological water bath with 95% O₂-5% CO₂ at 37°C for 3 h. The muscle strips were washed with fresh buffer each 15 min for the first hour and then left undisturbed for the subsequent 2 h. Strips were then contracted with 1 μM serotonin. At 5 min after the serotonin dose, one strip was snap-frozen and pulverized in liquid nitrogen, and the other strip was relaxed with 1 μM ISO for 5 min or 10 μM FSK for 5 min. Strips were quick-frozen in the same manner along with an untreated control strip. All samples were stored at -80°C for later analysis using one- or two-dimensional gel electrophoresis.

Determination of HSP20 phosphorylation in bovine ASM tissue

Proteins from frozen muscle strips were extracted in buffer containing 8 M urea, 10 mM DTT, and 4% CHAPS (UDC buffer). The mixtures were vortexed at RT overnight and then centrifuged at 14,000 rpm for 15 min. For one-dimensional separation, equal amounts (20 μg) of proteins were placed in Laemmli sample buffer (Bio-Rad) containing 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, heated for 5 min at 100°C, and separated by SDS-PAGE as described above. For two-dimensional analysis, 75 μg of extracted proteins were separated by first dimension isoelectric focusing (IEF) using the Protean IEF Cell (Bio-Rad), and second dimension using SDS-PAGE and probed with anti-HSP20 antibodies as described earlier (17).

Statistical analysis

Values are reported as means ± SE. Statistical analysis was performed by unpaired Student's *t*-test or one-way ANOVA followed by Tukey's test (GraphPad Software, San Diego, CA).

The criterion for significance was $P < 0.05$. Stress values are calculated with the following formula: Stress [newtons (N)/m²] = force (g) $\times$ 0.0987/area, where area is equal to the wet weight (milligrams)/length (millimeters at maximal length) divided by 1.055.

Results

Inhibition of PKA inhibits ISO-induced phosphorylation of HSP20 in HASM cells

To determine whether PKA mediates phosphorylation of HSP20 in ASM, HASM cells stably expressing either GFP (control) or the PKI-GFP chimera were generated as described previously (22). VASP phosphorylation by PKA at Ser157 causes a significant mobility shift on one-dimensional SDS-PAGE gels (8) and was used as a marker of intracellular PKA activity in the HASM cells (22). GFP and PKI HASM cells were stimulated with the β -agonist ISO, and the phosphorylation of VASP and HSP20 was determined by immunoblotting. Treatment with 1 μ M ISO for 10 min increased VASP phosphorylation in the control GFP cells but not in PKI-GFP cells (Fig. 1, A and B). Similarly, ISO led to increases in the phosphorylation of HSP20 in the control GFP cells but not in PKI-GFP cells (Fig. 1, A and C), suggesting the inhibition of PKA in PKI HASM cells. FSK (10 μ M, 10 min), which activates PKA through direct activation of adenylyl cyclase, also led to increases in HSP20 and VASP phosphorylation in GFP cells and not in PKI-GFP cells (Fig. 1, A–C).

PKA inhibition and cofilin dephosphorylation in HASM cells

Cofilin is an actin-depolymerizing protein that is activated upon dephosphorylation by phosphatases such as slingshot and chronophin (20,38). Phosphorylated cofilin contains a binding motif for the scaffolding protein 14-3-3, and association of cofilin with 14-3-3 protects cofilin from dephosphorylation (21). HSP20 also contains a binding motif for 14-3-3, and binding of 14-3-3 protein to phosphopeptide analogs of HSP20 prevents the association of cofilin with 14-3-3 (Ref. 16). Increases in cAMP levels have been demonstrated to cause cofilin dephosphorylation (51). To assess the role of PKA in the regulation of cofilin phosphorylation in ASM, GFP and PKI-GFP HASM cells were treated with either 1 μ M ISO for 10 min or 10 μ M FSK for 10 min, and phosphorylation of cofilin was analyzed by immunoblotting. FSK or ISO treatment significantly decreased phospho-cofilin levels in control GFP cells but had no effect in PKI-GFP cells (Fig. 1, A and D).

Inhibition of PKA prevents changes in morphology and focal adhesion complexes in HASM cells

To investigate the role of PKA-mediated phosphorylation of HSP20 in the regulation of central actin stress fibers, serum-starved HASM cells were treated with ISO or FSK, and stress fiber formation was analyzed by fluorescence microscopy. Treatment with 1 μ M ISO for 30 min or 10 μ M FSK for 30 min led to disruption of stress fibers, with only cortical actin remaining, in HASM cells that express GFP alone (Fig. 2, B and C). However, ISO or FSK treatment did not lead to stress fiber disruption in PKI-GFP-expressing cells (Fig. 2, E and F).

Loss of stress fibers has been associated with a loss of focal adhesion complexes (2). To examine the effect of PKA on focal adhesion complexes, HASM cells were treated with FSK (10 μ M, 30 min), and loss of focal adhesions were measured using interference reflection microscopy. Hep I peptide (from thrombospondin-1 and -2 with focal adhesion-labilizing activity; Ref. 36) was used as a positive control. Hep I treatment led to decreases in focal adhesions in both GFP and PKI-GFP cells (Fig. 3A). Hep I activates cGMP pathways leading to activation of PKG (37). PKG is not inhibited by PKI. FSK stimulation led to decreases in focal adhesions in GFP cells but not in PKI-GFP cells (Fig. 3A).

To examine the role of HSP20 phosphorylation in the regulation of cell morphology and focal adhesion disassembly, HASM cells were treated with a transducible synthetic phosphopeptide analog of HSP20 containing the active phosphorylation site of HSP20 (serine 16, which is phosphorylated during active relaxation; Ref. 4; WLRRApSAPLPGLK) attached to a protein transduction domain (YARAAARQARA) or a control peptide where phosphoserine is replaced with alanine and stress fiber formations and focal adhesion disassemblies were measured. Treatment with the phospho-HSP20 peptides led to changes in morphology, loss of stress fibers, and decreases in focal adhesion complexes (Fig. 3, A–G) in both GFP- and PKI-GFP-expressing cells. There were no changes in morphology, stress fibers, or focal adhesion complexes in cells treated with the peptide controls. To directly assess the role of phospho-HSP20 peptides in the regulation of cofilin phosphorylation, GFP and PKI-GFP HASM cells were treated with 50 μ M phospho-HSP20 peptides or control peptides for either 30 min or 24 h and phosphorylation of cofilin was analyzed by immunoblotting. Phospho-HSP20 peptide treatment significantly decreased phospho-cofilin levels in control GFP as well as in PKI-GFP cells (Fig. 3, H and I). There were no decreases in phospho-cofilin levels in cells treated with the HSP20 control peptides (Fig. 3I). Taken together, these data suggest that the effects of the phospho-HSP20 peptides are downstream of PKA.

Stimulation of HSP20 phosphorylation with ISO in intact bovine ASM

To confirm that phosphorylated HSP20 plays a role in PKA-induced ASM relaxation, isolated intact strips of bovine ASM were equilibrated in a muscle bath. The strips were precontracted with serotonin (5-hydroxytryptamine, 5-HT) and then treated with ISO, with force generation continuously recorded. Parallel strips were similarly treated and snap-frozen, and phosphorylation of HSP20 was subsequently analyzed by immunoblotting. Serotonin treatment led to a dose-dependent increase in force, and ISO induced a dose-dependent relaxation in bovine ASM (Fig. 4, A and B). When related to 110 mM KCl-induced contraction, 0.1, 1, and 10 μ M serotonin generated $6.3\% \pm 1\%$, $25.1\% \pm 5.5\%$, and $62.7\% \pm 15.4\%$, respectively. Two-dimensional gel electrophoresis and Western blot analysis demonstrate increases in the phosphorylation of HSP20 in response to ISO stimulation (Fig. 4C).

To examine the direct role of HSP20 phosphorylation on ASM relaxation, bovine ASM strips were precontracted with either serotonin (1 or 5 μ M) or increasing doses of carbachol (0.1, 0.5, and 5 μ M) and treated with phospho-HSP20 peptides or HSP20 control peptides. Transduction of serotonin precontracted strips of bovine ASM with phospho-HSP20 peptides led to a dose-dependent decrease in stress, which was significant at 2 mM phospho-HSP20 peptide ($45.7\% \pm 7.6\%$ compared with $90.0\% \pm 1.4\%$), whereas there was no significant decrease in stress with HSP20 control peptides (Fig. 4D). The decreases in stress generated by the phospho-HSP20 peptide with respect to maximum KCl contraction were $41.5\% \pm 3.6\%$, $39.2\% \pm 1.7\%$, $35.7\% \pm 1.7\%$, $29.3\% \pm 3.2\%$, and $20.2\% \pm 2.4\%$ for 0, 0.1, 0.5, 1, and 2 mM phospho-HSP20 peptide, respectively. Similarly, phospho-HSP20 peptides, and not the control peptides, led to a dose-dependent decrease in stress in carbachol precontracted strips (Fig. 5, A–C). However, the peptide analogs did not lead to complete relaxation of bovine ASM. Phospho-HSP20 peptides were more effective on strips contracted with lower concentration of carbachol (0.1 μ M) that generated 40–60% of KCl contraction compared with the higher concentration (0.5 or 5 μ M) that generated 80–120% of KCl contraction. When bovine ASM was contracted with 0.5 μ M carbachol, significant relaxation was achieved only at 4 mM phospho-HSP20 peptide compared with control peptide ($75.11\% \pm 3.5\%$ and $97.63\% \pm 3.4\%$, respectively), whereas at 0.1 μ M carbachol, significant relaxation was achieved at 3 and 4 mM phospho-HSP20 peptide ($55.57\% \pm 9.7\%$ and $42.5\% \pm 12.3\%$ for 3 and 4 mM HSP20 peptide compared with $96.9\% \pm 3.0\%$ and $97.6\% \pm 3.4\%$ for 3 and 4 mM control peptide). There was no significant difference in the response of the control peptide to 0.1, 0.5, or 5 μ M carbachol (data not shown).

To determine whether the activation of PKA led to changes in cofilin phosphorylation, bovine ASM strips were precontracted with 5 μ M serotonin and treated with 1 μ M ISO or 10 μ M FSK for 5 min, and the phosphorylation of cofilin was analyzed by SDS-PAGE and Western blotting. Activation of PKA by ISO or FSK led to a significant decrease in the phosphorylation of cofilin (Fig. 6, A and B).

Discussion

In this investigation, we used cultured HASM cells that expressed a peptide inhibitor of PKA to assess events downstream of PKA signaling. Treatment of HASM cells with activators of PKA led to increases in the phosphorylation of the PKA substrates VASP and HSP20 in cells expressing GFP alone but not in cells expressing PKI-GFP. This was associated with distinct morphological changes, decreases in actin stress fibers, and focal adhesion complexes in the GFP but not in PKI-GFP cells. There were decreases in the phosphorylation of the actin-depolymerizing protein cofilin in the GFP but not in PKI-GFP cells. Hence, one possible mechanism to explain the loss of actin stress fibers and focal adhesions is activation of cofilin. PKA inhibition has been demonstrated earlier in HASM cells expressing PKI, where treatment with ISO or FSK caused only a small percentage of VASP to shift relative to that observed for the GFP-expressing lines, and agonist-stimulated cAMP response element (CRE)-luciferase activity in these cells was abolished (22).

In this study, we demonstrate that the phosphopeptide analogs of HSP20 lead to loss of stress fibers, decreases in focal adhesion complexes, and decreases in cofilin phosphorylation in both GFP- and PKI-GFP-expressing HASM cells. These data suggest that the events upstream of PKA can be bypassed by directly introducing phosphopeptide analogs of at least one of the substrates of PKA and that the mechanism of action may be competition for binding to the scaffolding protein 14-3-3. However, the effect of phospho-HSP20 peptides on cofilin dephosphorylation was more pronounced in PKI-GFP compared with GFP-expressing cells, suggesting that other pathways may also be involved in dephosphorylation of cofilin or that the expression of PKI led to alterations in the expression or activity of other signaling events. ISO-induced actin depolymerization in ASM cells has been reported earlier to be by PKA-dependent as well as PKA-independent pathways involving Src kinase and G_s (24,25). Cofilin is an actin-depolymerizing protein that, when phosphorylated, binds to the intracellular scaffolding protein, 14-3-3 (Refs. 5,21). When displaced from 14-3-3, cofilin becomes dephosphorylated and activated as an actin-depolymerizing protein (20,38). Binding of 14-3-3 protein to phosphopeptide analogs of HSP20 prevents the association of cofilin with 14-3-3 (Ref. 16). It is likely that cyclic nucleotide-dependent relaxation of smooth muscles includes multiple and redundant pathways and mechanisms.

β -Agonist- or nitrovasodilator-induced phosphorylation of HSP20 has been demonstrated to mediate relaxation of vascular smooth muscle from various tissues (4,17,18,43). Phosphorylation of HSP20 also inhibits agonist-mediated contraction, intimal hyperplasia, and platelet aggregation (33,53,57). Here, we have also demonstrated that ISO-induced relaxation of intact bovine ASM is associated with phosphorylation of HSP20 (Fig. 4C). Phosphopeptide analogs of HSP20 also relaxed bovine ASM, demonstrating a direct role for HSP20 in ASM relaxation (Figs. 4D and 5). However, the analogs did not completely relax ASM. This may be due to the fact that the phosphopeptide analogs do not contain the complete structure of the HSP20 molecule and hence require higher concentrations than the intact, phosphorylated HSP20 molecule (17). Another possibility is that PKA phosphorylates other proteins to enable more complete relaxation. For example, β_2 -adrenergic relaxation of ASM has been associated with PKA-dependent and -independent regulation of large-conductance, calcium-activated potassium channels resulting in hyperpolarization as well as mechanisms involving calcium sensitivity of the contractile elements due to activation of myosin light chain phosphatase

(31). The effect of ISO in nonhuman airways has also been demonstrated to involve enhanced Ca^{2+} pump activity to decrease $[\text{Ca}^{2+}]_i$ and myosin light chain phosphatase activation to decrease Ca^{2+} sensitivity of the contractile apparatus (27). Activation of PKA by ISO or FSK led to a significant decrease in the phosphorylation of cofilin in bovine ASM. These results suggest that ISO-induced PKA-mediated phosphorylation of HSP20 may induce actin depolymerization through cofilin dephosphorylation in ASM, and this may be one of the mechanisms for Ca^{2+} -independent relaxation of ASM along with the other mechanisms such as Ca^{2+} desensitization and myosin phosphatase activation.

β -Agonists are the most commonly used therapy for relief of acute bronchospasm in asthmatics. It is widely assumed that β -agonists mediate their effect primarily by increasing cAMP concentration through activation of β_2 -adrenergic receptor-adenylyl cyclase pathway. Several mediators of inflammation and therapies such as β -agonists themselves can cause alterations in β_2 -adrenergic receptor responsiveness in a time-dependent and cell-specific manner (42). Agonist-specific desensitization may also be responsible for the loss of prophylactic bronchoprotection and deterioration of asthma control clinically observed with regular use of β -agonists (13,46,49). Several investigations have also identified that airway inflammation or cytokine treatment contributes to β_2 -adrenergic receptor dysfunction and a loss of the relaxant effect of β -agonists in ASM cells, tissues, and in vivo models (9,23,34,35). Adverse effects have been documented with inhaled β_2 -adrenergic agonist in patients with a genetic polymorphism that results in homozygosity for arginine rather than glycine at amino acid residue 16 of the β_2 -adrenergic receptor (26). A recent meta-analysis that compares anticholinergics and β_2 -adrenergic agonists indicated that treatment with β_2 -adrenergic agonists led to a twofold increase in respiratory deaths (44). Collectively, these studies suggest that regulation of proximal transmembrane signaling events in the β_2 -adrenergic receptor- G_s -adenylyl cyclase pathway may limit the efficacy of β -agonist therapy. In the present study, we demonstrate that β -agonist-induced relaxation of ASM is associated with increases in the phosphorylation of HSP20. In addition, phosphopeptide analogs of HSP20 linked to a transduction domain can produce the same morphological and physiological responses as do activation of the cAMP-PKA pathway. Thus transducible peptide analogs of HSP20 that act in the same manner as the physiological downstream PKA effector HSP20 represent a potential treatment approach to bronchospasm capable of overcoming problems associated with β_2 -adrenergic receptor desensitization.

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Fig. 1.

Inhibition of PKA inhibits isoproterenol (ISO)- or forskolin (FSK)-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) and small heat shock-related protein HSP20 and dephosphorylation of cofilin in human airway smooth muscle (HASM) cells. HASM cell lines were treated with 1 μ M ISO (A; lanes 2 and 4) or 10 μ M FSK (A; lanes 6 and 8) for 10 min and snap-frozen. Whole cell lysates (20 μ g) of control and ISO- or FSK-treated cells expressing green fluorescent protein (GFP; lanes 1, 2, 5, and 6) or GFP-PKA inhibitory fusion protein (PKI-GFP; lanes 3, 4, 7, and 8) were separated by SDS-PAGE and probed with the indicated antibodies. The positions of VASP and phosphorylated VASP (pVASP) are indicated by arrows on the left. Bands were normalized to actin, and ratios of pVASP-to-VASP (B), pHSP20-to-HSP20 (C), and phospho-cofilin-to-cofilin (D) were determined, $n = 4-6$; * $P < 0.05$ with respect to control.

Fig. 2.

Inhibition of the PKA inhibits ISO- or FSK-mediated stellation and stress fiber disruption in HASM cells. *A–F*: representative epifluorescence micrographs of HASM control (*A* and *D*), 1 μ M ISO for 30 min (*B* and *E*), or 10 μ M FSK for 30 min (*C* and *F*) cells expressing GFP (*A–C*) or PKI-GFP (*D–F*). Cells grown for 24 h in serum-free medium were treated with the agents and then washed, processed for microscopy, and labeled with Alexa 568-conjugated phalloidin to reveal the actin cytoskeleton and 4,6-diamidino-2-phenylindole (DAPI) to reveal nucleus; bar = 50 μ m.

Fig. 3.

pHSP20 peptide causes stress fiber disruption and focal adhesion (FA) changes in GFP- as well as PKI-GFP-expressing HASM cells. HASM cells were grown as described in Materials and Methods. **A:** cells were serum-starved overnight and either untreated (control) or treated with 100 nM Hep I (thrombospondin peptides, positive control), 10 μ M FSK, or 25 μ M pHSP20 peptide (HSP20 peptide) containing a protein transduction domain and the active phosphorylation site of HSP20 or HSP20 control peptide (HSP20 control) for 30 min. Cells were fixed and examined (at least 250 cells per condition) for the presence or absence of FA by interference reflection microscopy. The percentage of cells positive for FA was determined in 5 independent experiments ($n = 5$; $*P < 0.05$). **B–G:** representative epifluorescence micrographs of HASM control (**B** and **E**), 50 μ M pHSP20 peptide (**C** and **F**), and 50 μ M HSP20 control peptide (**D** and **G**) for 24 h in cells expressing GFP (**B–D**) or PKI-GFP (**E–G**). Cells grown for 24 h in serum-free medium were treated with the agents and then washed, processed for microscopy, and labeled with Alexa 568-conjugated phalloidin to reveal the actin cytoskeleton and DAPI to reveal nucleus; bar = 50 μ m. **H:** HASM cell lines were untreated (C; lanes 1 and 4) or treated with 50 μ M pHSP20 peptide for 30 min or 24 h (lanes 2, 3, 5, and 6) and snap-frozen. Whole cell lysates (20 μ g) of control and treated cells expressing GFP (**H**; lanes 1–3) or PKI-GFP (**H**; lanes 4–6) were separated by SDS-PAGE and probed with the indicated antibodies. **I:** densitometric measurements of the bands, which were normalized to GAPDH, and ratio of phospho-cofilin-to-cofilin were determined and plotted along with results obtained from treatment of GFP and PKI-GFP cells with HSP20 control peptides. $n = 3$; $*P < 0.05$ with respect to control.

Fig. 4.

HSP20 phosphopeptide treatment leads to relaxation of bovine ASM. Isolated bovine ASM strips were suspended in a muscle bath and equilibrated for 2 h in bicarbonate buffer as described in Materials and Methods. The muscle strips were treated with cumulative log doses (0.01–1 μM) of serotonin (Ser) followed by cumulative log doses (0.01–1 μM) of ISO, and force generated was recorded. A representative tracing for force generation with Ser and ISO is shown (A). Cumulative data were obtained when the force was converted to stress, and decrease in stress minus that of the untreated control was converted to a percentage of the maximal Ser (1–5 μM) contraction and plotted against the concentration of ISO (means $\pm$ SE, $n = 3$; * $P < 0.05$; B). Bovine ASM strips were equilibrated in a water bath and contracted with

1 μ M Ser for 5 min, followed by 1 μ M ISO for 5 min. The strips were quick-frozen and pulverized, and proteins were analyzed by 2-dimensional (2-D) gel electrophoresis and immunoblotting. Representative section of 2-D blot (pH 4–7) is shown (C) with untreated control, Ser-treated tissue, and Ser + ISO. Arrow indicates phosphorylation of HSP20 (pHSP20). These blots are representative of 3 separate experiments. D: bovine ASM strips were equilibrated in a muscle bath as described above and precontracted with 5 μ M Ser. The strips were then relaxed with increasing doses (0.1–2 mM) of pHSP20 peptide (P20 peptide) or HSP20 control peptide analogs. The percent contraction was calculated as above and plotted against HSP20 peptide or control peptide concentration (means $\pm$ SE, $n = 3$; $*P = 0.034$ at 2 mM P20 peptide compared with 2 mM control peptide).

Fig. 5.

HSP20 phosphopeptide treatment leads to relaxation of carbachol (CCh)-contracted bovine ASM. *A* and *B*: bovine ASM strips were equilibrated in a muscle bath as described in Fig. 4 legend and precontracted with increasing doses of CCh (“a” = 0.01 μ M, “b” = 0.05 μ M, and “c” = 0.1 μ M). The strips were then relaxed with increasing doses (“d” = 0.5 mM, “e” = 1 mM, “f” = 2 mM, “g” = 3 mM, and “h” = 4 mM) of P20 peptide analogs or HSP20 control (P20 control) peptide analogs. *C*: cumulative data obtained from the effect of P20 peptide when bovine ASM strips were contracted with 0.1, 0.5, or 5 μ M doses of CCh. The percent contraction was calculated as in Fig. 4 and plotted against pHSP20 peptide or HSP20 control peptide concentration (means $\pm$ SE, $n = 3$ for each concentration of CCh; * $P < 0.001$ when P20 control is compared with 0.1 μ M + 3 and 4 mM P20 peptide and $P < 0.05$ when P20 control is compared with 0.5 μ M + 4 mM P20 peptide).

Fig. 6.

Isoproterenol (IPT) and FSK reduce the levels of phospho-cofilin in bovine ASM. Isolated bovine ASM strips were suspended in a muscle bath and equilibrated for 3 h in bicarbonate buffer as described in Materials and Methods. Bovine ASM strips were contracted with 5 μ M Ser for 5 min, 5 μ M Ser for 5 min followed by 1 μ M IPT, or 5 μ M Ser for 5 min followed by 10 μ M FSK for 5 min. The strips were quick-frozen and pulverized, and the proteins were separated by SDS-PAGE and probed with the indicated antibodies. A shows a representative blot. Bands were normalized to β -actin, and ratio of phospho-cofilin-to-cofilin (B) was determined; $n = 3$; * $P < 0.05$ with respect to Ser alone. 5-HT, 5-hydroxytryptamine.