The small heat shock-related protein 20 (HSP20) is present in four isoforms in bovine carotid artery smooth muscles. Three of the isoforms are phosphorylated and one is not. Increases in the phosphorylation of two isoforms of HSP20 (isoform 3, pI 5.9; and 8, pI 5.7) are associated with cyclic nucleotide-dependent relaxation of bovine carotid artery smooth muscles. Increases in the phosphorylation of another isoform (isoform 4, pI 6.0) are associated with phorbol ester-induced contraction of bovine carotid artery smooth muscles. In this investigation we determined that isoforms 3 and 8 are phosphorylated on Ser16 of the HSP20 molecule during activation of cAMP-dependent signaling pathways. Phosphorylation site-specific antibodies produced against a peptide containing phosphorylated Ser16 recognized isoforms 3 and 8 but not isoform 4. In human vascular tissue, only isoform 3 is present. Incubation of transiently permeabilized strips of bovine carotid artery smooth muscle with synthetic peptides in which Ser16 is phosphorylated, inhibits contractile responses to high extracellular KCl and to serotonin. These data suggest that phosphorylation of HSP20 on Ser16 modulates cAMP-dependent vasorelaxation.

A major phosphorylation event that occurs with cyclic nucleotide-dependent relaxation of vascular smooth muscle is an increase in the phosphorylation of two 20-kDa proteins (isoform 3, pI 5.9; and 8, pI 5.7) (1–3). We recently identified these 20-kDa phosphoproteins as different phosphorylated forms of a small heat shock-related protein, HSP201 (4). In addition, HSP20 can be phosphorylated in vitro by both cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) (4). HSP20 is also phosphorylated during endothelial-dependent vasorelaxation of isolated segments of bovine carotid artery smooth muscle (5).

In a vascular smooth muscle, umbilical artery smooth muscle, that is refractory to cyclic nucleotide-dependent vasorelaxation, there is no significant increase in the phosphorylation of HSP20 in response to activation of PKA or PKG (2). HSP20 is present in umbilical artery smooth muscle and can be phosphorylated by PKA in vitro using homogenates of umbilical smooth muscle (6). Taken together, these data support a role for phosphorylated HSP20 in mediating cyclic nucleotide-dependent vasorelaxation.

Histamine and phorbol ester-induced contractions of bovine carotid artery smooth muscle are associated with an increase in the phosphorylation of another 20-kDa protein (isoform 4, pI 6.0) (1). The subsequent activation of cyclic nucleotide-dependent signaling pathways leads to a decrease in the phosphorylation of isoform 4. This 20-kDa protein is immunoreactive with specific polyclonal antibodies raised against HSP20 (4). Thus, increases in the phosphorylation of this isoform of HSP20 are associated with smooth muscle contraction and decreases are associated with activation of cyclic nucleotide-dependent signaling pathways.

The purpose of this investigation was to determine the specific site on the HSP20 molecule that is phosphorylated during cyclic nucleotide-dependent vasorelaxation. We demonstrated that Ser16 is the site phosphorylated on HSP20 by PKA or PKG. We then prepared peptides in which the Ser16 site was altered and determined whether these peptides altered the contractile responses of transiently permeabilized vascular smooth muscle.

EXPERIMENTAL PROCEDURES

Materials—Human skeletal muscle HSP20 was purified as described previously (7). The catalytic subunit of cAMP-dependent protein kinase (PKA) and modified trypsin, sequence grade, was purchased from Promega (Madison, WI). Hepes was obtained from American Bioanalytical (Natick, MA). Urea, SDS, glycerine, and Tris were from Research Organics (Cleveland, OH). Coomassie Brilliant Blue was from ICN Biomedicals Inc (Aurora, OH). [γ-32P]ATP and [32P]orthophosphate were from Amersham Pharmacia Biotech. Forskolin and 3-isobutyl-1-methanethione (IBMX) were from Calbiochem. The inhibitor of PKA, PKI, was purchased from Peninsula (Belmont, CA). Pipерazine diacidamide and other electrophoresis reagents were from Bio-Rad. CHAPS, EGTA, EDTA, polyoxyethylene-sorbitan monolaurate (Tween 20) and all other reagent grade chemicals were from Sigma. Purified cGMP-dependent protein kinase (PKG) was obtained from Dr. Tom Lincoln (University of Alabama, Birmingham, AL). Polyclonal antibodies against HSP20 were produced as described previously (7), against α-crystallin were from Upstate Biotechnology (Lake Placid, NY), and antibodies against myotonic kinase binding protein (MKBP) were from Dr. Atsushi Suzuki (Yokohama, Japan). Goat anti-rabbit secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Protein concentrations
were determined using the Coomassie Plus Protein Assay Reagent (Pierce).

Preparation of Vascular Smooth Muscle Strips—Intact bovine carotid arteries were obtained from an abattoir (Shapiro’s meatpackers, Augusta, GA). Human aortic tissues were obtained from organ donors with approval from the Emory University School of Medicine and the Emory University Institutional Review Board. The adventitia was dissected from the arteries, and the endothelial lining was denuded with a cotton-tipped applicator. The arteries were opened longitudinally, and thin transverse strips were cut. Vessel viability was determined by concurrent muscle bath experiments as described previously (2).

In Vitro Phosphorylation of HSP20—HSP20 was phosphorylated in a reaction mixture containing 20 μm Tris (pH 7.4), 10 mM magnesium acetate, 100 mM of the catalytic subunit of cAMP-dependent protein kinase or 100 mM of cGMP-dependent protein kinase. For experiments using PKG, the peptide inhibitor of PKA (PKI, 1 μM, final concentration), was added. The reaction was initiated with the addition of 200 μM [γ-32P]ATP (800 cpm/pmol) and incubated for 30 min at 30 °C. The reaction was stopped by the addition of 1.1,1,1-trifluoroacetone (6.25 mM Tris (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% bromphenol blue and boiled for 5 min. The proteins were separated on 15% polyacrylamide SDS gels, fixed in 10% trichloroacetic acid, and stained with Neuhoff’s Coomassie stain (10% ammonium sulfate, 2.4% phosphoric acid, 0.1% Coomassie Brilliant Blue G-250, 20% methanol) (8).

In Situ Phosphorylation of HSP20—Strips of bovine carotid artery smooth muscle were incubated in 150 μCi/ml [32P]orthophosphate in 10 mM Hepes (pH 7.4), 150 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO4, 1.0 mM NaH2PO4, 10 mM glucose, 5.5 mM Na2HCO3 and oxygenated with with 95% O2, 5% CO2 at 37 °C for 4 h. The muscle strips were then stimulated with forskolin (10 μM) for 10 min and then ischemia (1 min) and forskolin (10 μM) for 10 min, 15% peroxide and boiled for 5 min. The proteins were separated on 15% polyacrylamide SDS gels, fixed in 10% trichloroacetic acid, and stained with Neuhoff’s Coomassie stain, and then separated on 12% acrylamide SDS gels (14). The gels were fixed in 10% trichloroacetic acid and stained with 10% acetic acid, 10% dithiothreitol. The suspension was quickly frozen in liquid nitrogen and then allowed to return to room temperature. The suspension was centrifuged (10,000 × g) and washed three times in acetone. The pellets were dried under a stream of nitrogen and solubilized in 9 M urea, 2% CHAPS, and 100 mM dithiothreitol overnight at room temperature.

Two-dimensional Gel Electrophoresis—The isolated phosphoproteins were separated by two-dimensional gel electrophoresis using the method of O’Farrell (9) modified by Hochstrasser et al. (10). In brief, 5 mg of protein was loaded onto 12 × 15-cm slab isofocusing gels consisting of 4% acrylamide, 0.1% piperazine diacrylamide, 9 M urea, 5% ammonium (5 parts 6–8, 3 parts 5–7, and 2 parts 3–10), and 2% CHAPS. Temed (0.04%) and ammonium persulfate (0.1%) were used to initiate polymerization. The cathode buffer consisted of 20 mM sodium hydroxide and the anode buffer 10 mM phosphoric acid. The proteins were focused for 10,000 V h. The gels were fixed in 10% trichloroacetic acid and stained with Neuhoff’s Coomassie stain (9), and the lanes of stained proteins were cut from the gels and equilibrated in 10 mM Tris (pH 6.8), 3% SDS, 19% ethanol, 4% β-mercaptoethanol, and 0.004% bromphenol blue for 10 min. The proteins were then separated on 12% acrylamide SDS gels (14). The gels were fixed in 10% trichloroacetic acid, stained with Neuhoff’s Coomassie stain, and the spots corresponding to the specific isoforms of HSP20 were excised from the gels.

In Gel Tryptic Digests and Amino Acid Sequencing—The gel pieces containing the specific isoforms of HSP20 were digested by the in gel tryptic digest method of Hellman et al. (11). In brief, the gel pieces were destained in 40% methanol and washed in 0.2 m ammonium bicarbonate (pH 8.9), 50% acetonitrile. The gel pieces were then dried under a stream of nitrogen and reconstituted in 0.2 m ammonium bicarbonate (pH 8.9), 50% acetonitrile, 0.02% Tween 20. The proteins were digested with the addition of 0.5 μg of trypsin for 12 h. The proteolytic fragments were extracted with 60% acetonitrile, 0.1% trifluoroacetic acid. The peptides were separated with reverse phase narrow-bore liquid chromatography on a C-18 column using a 260-min gradient of 0–40% acetonitrile in 0.065 to 0.05% trifluoroacetic acid at a flow rate of 100 μl/min, with the Smart System (Pharmacia Biotech, Uppsala, Sweden). 50-μl fractions were collected and each well was dried. The peptides were separated by mass spectrometry.

Peptide Sequencing—Peptides from the Smart system were applied to a ProSorb membrane (Perkin-Elmer and Applied Biosystems) as per the manufacturers’ directions. The peptides were sequenced on a Procise (Applied Biosystems, model 492) instrument using standard protocols.

Phosphopeptide Mapping—Phosphopeptide mapping was performed accord-
were again washed three times and subsequently incubated with antirabbit antibodies conjugated to alkaline phosphatase (Promega, Madison, WI) for 2 h at room temperature. The plates were washed three times and developed with alkaline phosphatase substrate (Sigma 104 phosphatase substrate). The optical density was read at 405 nm.

**Transient Permeabilization of Isolated Strips of Vascular Smooth Muscle**—Fine strips of bovine carotid artery smooth muscle (0.1 mm wide × 8 mm long) were cut with a razor blade under a dissecting microscope and permeabilized using a protocol that has been modified to introduce the 21-kDa photoprotein, aequorin (15–18). The strips were washed 3 times in stripping solution, 25 mM HEPES, 120 mM KCl, 5.6 mM glucose, 0.2% bovine serum albumin, and 3 mM EGTA and then incubated in stripping solution for 30 min at room temperature while gently shaken. The strips were then incubated in the stripping solution with specific peptides for another 30 min on ice. Calcium was added directly to the solution in three increments, 5 min apart to a final concentration of 1 mM. It has been determined that a 13-kDa molecule with a radioactive tag is not released after this protocol (18) suggesting that the cell membranes regain their integrity.

**Physiologic Contractile Responses**—The strips were tied under magnification at each end with a 7–0 prolene suture (Johnson and Johnson, Cincinnati, OH) and suspended in a muscle bath in Heps solution (10 mM Heps, 140 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO4, 1.0 mM NaH2PO4, 1.0 mM CaCl2, 10 mM glucose (pH 7.4)) at room temperature. The muscle strips were placed under 0.5 g of tension and allowed to relax to a baseline tension over 15 min. The strips were fixed at one end to a stainless steel wire and attached to a Kent Scientific (Litchfield, CT) force transducer (TRN001) interfaced with a Data Translation A-D board, DT2801 (Data Translation, Inc., Marlboro, MA). Data were acquired with Lab Tech Notebook software (Laboratory Technologies Corp., Wilmington, MA). Agonists were added directly to the bath.

**In Vitro Phosphorylation of Synthetic Peptides**—The peptides (200 μg) were phosphorylated in a 50-μl reaction mixture containing 20 mM Tris (pH 7.4), 10 mM magnesium acetate, 5 mM KPO4, 5 mM EDTA, 2 mM 2-mercaptoethanol, 6 units (15 nM) of the catalytic subunit of PKA. The reactions were initiated with the addition of 200 μM [γ-32P]ATP (800 cpm/nmol) and incubated for 15 min at 30 °C, terminated by spotting 20-μl aliquots onto phosphocellulose papers (Whatman P81). The papers were washed three times in ice-cold 75 mM phosphoric acid, rinsed in acetone, and allowed to air dry. The papers were counted in a scintillation counter (Beckman, Irving, CA).

**Data Analysis**—Values are reported as mean ± S.E., and n refers to the number of animals examined. The statistical difference between the two groups was determined with Student’s t test and between multiple groups with one-way repeated measures analysis of variance using Sigma Stat software (Jandel Scientific, San Rafael, CA). A P value less than 0.05 was considered significant. Densiometric analysis was performed with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**Identification of the Phosphorylation Sites on the HSP20 Molecule**—Phosphorylation of purified rat skeletal muscle HSP20 with the catalytic subunit of PKA resulted in two proteolytic fractions that contained radioactive counts (Fig. 1). The amino acid sequence obtained from the major proteolytic fragment (peak 1, fraction 19) was XAXXPLPGLSAPGRQR and that from the minor fragment (peak 2, fractions 27 and 28) was APSVALPQAVPQTPDG. These peptides displayed significant homology with the known amino acid sequence of human HSP20 (fraction 19, 70% homology, and fraction 27/28, 100% homology) (7). The fragment from peak 1 had a PKA consensus sequence (RRAS) corresponding to Ser16 on the HSP20 molecule. The fragment from peak 2 had a less suitable PKA consensus sequence (RAPS) corresponding to Ser16 on the HSP20 molecule. Phosphorylation of purified rat skeletal muscle HSP20 with PKG resulted in one fraction that contained the majority of the radioactive counts. This fraction had a similar mobility on the column as the major peak obtained after phosphorylation with PKA (fraction 19). Peptide analysis was not performed on this fraction.

To determine the sites on the HSP20 molecule that are phosphorylated when intact strips of muscle are stimulated with substances that activate cyclic nucleotide-dependent signaling pathways, strips of bovine carotid artery smooth muscle were incubated with [32P]orthophosphate and stimulated with forskolin (10 μM) and isobutylmethylxanthine (IBMX, 1 mM) for 10 min. This combination of an adenylate cyclase activator, forskolin, and a phosphodiesterase inhibitor, IBMX, led to the maximal phosphorylation of HSP20 (data not shown). The proteins were separated with two-dimensional gel electrophoresis. Autoradiography revealed two 20-kDa spots that were immunoreactive with antibodies against HSP20 (Fig. 2, immunoblots not shown). The protein that had been previously described as isoform “3” with a pl of 5.9 was digested, and the proteolytic fragments were separated by reversed phase fast protein liquid chromatography. The peak of radioactivity was again in fraction 19, with a minor component of counts in fractions 27 and 28 (Fig. 2B). The proteolytic fragment from fraction 19 contained the amino acid sequence RAAXXLPGGLSPGRX. This sequence had 100% homology to the known sequence of human HSP20 and with the peptide isolated from fraction 19 after the in vitro phosphorylation of the purified HSP20. The RAXX likely represents RAS corresponding to Ser16 on the HSP20 molecule that was phosphorylated by PKA. We were unable to resolve the proteolytic fragment from fractions 27/28 for sequence analysis. The proteolytic fragment with the peak of radioactivity from isoform “8” (pl of 5.7) was again in fractions 27/28 (Fig. 2C). The proteolytic fragment from fraction 19 contained the amino acid sequence RASALPGGLSPGR (100% homology with human HSP20). This peptide again contained the consensus sequence for PKA phosphorylation: RRAS with 100% homology (7). The fragment from peak 19 (Fig. 2C) contained the amino acid sequence RAAXXLPGGLSPGRX. This sequence had 100% homology to the known sequence of human HSP20 and with the peptide isolated from fraction 19 after the in vitro phosphorylation of the purified HSP20. The RAXX likely represents RAS corresponding to Ser16 on the HSP20 molecule that was phosphorylated by PKA. We were unable to resolve the proteolytic fragment from fractions 27/28 for sequence analysis. The proteolytic fragment with the peak of radioactivity from isoform “8” (pl of 5.7) was again in fraction 19 (Fig. 2C). The proteolytic fragment from fraction 19 contained the amino acid sequence RASALPGGLSPGR (100% homology with human HSP20). This peptide again contained the consensus sequence for PKA phosphorylation: RRAS with Ser16 representing the phosphorylation site. The proteolytic fragment from fractions 27/28 had a sequence of LPGVDP-PAAVTSALSPG (100% homology with human HSP20), cor-
responding to the carboxyl terminus of the HSP20 molecule, and contained no consensus sites for PKA or PKG (Fig. 3).

To determine the phosphorylation site on the isoform with a pI of 6.0 (isoform 4), strips of bovine carotid artery smooth muscle were incubated in the presence of [32P]orthophosphate and stimulated with the phorbol ester, phorbol dibutyrate (PDBu, 1 μM, 45 min), and the proteins were separated by two-dimensional electrophoresis. The spot corresponding to isoform 4 was digested, and the proteolytic fragments were separated. The peak of radioactivity was in fractions 27 and 28 (Fig. 2E), and the peptide sequence from this fraction was RYRLPPGVPPAAVTSAL (94% homology with human HSP20). This sequence is found at the carboxyl terminus of the HSP20 molecule (amino acids 120–137).

The chromatographs of the peptides from in vitro phosphorylation of HSP20 with PKA (Fig. 4A) were similar to the chromatographs of isoform 3 after IBMX/FSK treatment (Fig. 4B). The peptide patterns on the chromatographs from isoforms 8 and 4 were also similar (Fig. 4, C and D). However, there were differences between the peptide patterns from isoform 3 and isoforms 4 and 8. Finally, phosphopeptide mapping of a strip of gel containing all three isoforms demonstrated that there were phosphorylated peptides unique to isoforms 4 and 8 (Fig. 4, E and F).

Characterization of Phosphorylation State-specific Polyclonal Antibodies—Affinity purified phosphorylation state-specific antibodies for phosphorylated HSP20 recognized only isoforms 3 and 8 of HSP20 that were phosphorylated by PKA (Fig. 5). On the other hand, a purified polyclonal antibody (7) recognized all isoforms of HSP20 including non-phosphorylated HSP20.

MEIRVPQVPSWLR/R/ASA/LPGFSTPG/R/LFDQRF/GE
MEIRVPQVPSWLR/R/ASA/LPGSAPGR/LFDQRF/GE
GLLEAEALCPAAIPYLR/APSVALPTAQVPTDPG
GLLEAEALCPITTLAPYLR/APSVALPTAQVPTDPG
YFSSVLV/DKV/HFSPEIEISVK/VVGDHV/EVHAR/HEE
HFSVLLV/DKV/HFSPEI/AVK/VVGEHV/EVHAR/HEE
RPDEI/IGF/R/YR/LPPGDPAVTSALSPEGV
RPDEI/IGF/R/YR/LPPGDPAVTSALSPEGV
LSIQATPSAQSLPSPPAAK: rat HSP20
LSIQAAPASQA--PPPAAKA: human HSP20

Fig. 3. Location of the HSP20 phosphorylation sites within the aligned amino acid sequences of human and rat HSP20. The sequences of human and rat HSP20 from Kato et al. (7) were aligned, and the tryptic digest sites are marked with a slash (/). The amino acid sequences of proteolytic fragments from specific column fractions are underlined. The in situ cAMP-dependent phosphorylation site is marked with a dashed box.

To determine the sensitivity of the phosphorylation state-specific antibodies, recombinant HSP20 was phosphorylated in vitro by the catalytic subunit of PKA. The phosphorylation state-specific antibodies recognized 3–100 ng of phosphorylated HSP20 in an ELISA (Fig. 6). By Western blotting, the antibodies recognized 1–10 μg of phosphorylated HSP20 in a linear fashion (Fig. 6).

To determine the specificity of the phosphorylation state-
specific antibodies for HSP20 in intact tissues, homogenates of bovine carotid artery smooth muscles (30 μg of protein) were treated with buffer alone (control), sodium nitroprusside (10 μM, 10 min), or forskolin (10 μM, 10 min) and then separated on SDS-PAGE and transferred to Immobilon. The blots were probed with the phosphorylation state-specific antibodies and subsequently re-probed with affinity purified polyclonal antibodies that recognize all isoforms of HSP20 (7). The affinity purified phosphorylation state-specific antibodies recognized a band at a relative mobility of 20 kDa in the sodium nitroprusside- and forskolin-treated tissues, whereas the affinity purified polyclonal antibody recognized three forms of HSP20 (Fig. 7).

The phosphorylation of an additional isoform of HSP20, pI 6.0, (isoform 4), increases with phorbol ester stimulation of carotid artery smooth muscle (1). The phosphorylation of isoform 4 decreases with activation of cyclic nucleotide-dependent signaling pathways. Radiolabeled strips of carotid artery smooth muscle were treated with phorbol dibutyrate (PDBu, 100 nM, for 45 min) followed by forskolin (10 μM, for 10 min). The strips were homogenized and the proteins separated by two-dimensional electrophoresis and transferred to Immobilon. The blots were exposed to x-ray film (autoradiographs) and subsequently probed with the phosphorylation state-specific antibodies. The phosphorylation state-specific antibodies recognized isoforms 3 and 8 but did not recognize isoform 4 (Fig. 8B). The blots were again probed with antibodies that recognize all isoforms of HSP20. Isoforms 3, 4, 8, as well as a nonphosphorylated pool of HSP20 were identified (Fig. 8C). Finally, the blots were probed with an antibody against another recently identified small heat shock protein myotonic dystrophy-binding protein (19). This antibody recognized a 20-kDa protein with a pI of 5.3 that was not phosphorylated in response to PDBu or forskolin treatment (Fig. 8A and D). This protein does not contain the RRAS16 site (19).

**Fig. 5. Characterization of phosphorylation state-specific antibodies.** HSP20 was purified from human skeletal muscle, and some of the protein was phosphorylated in vitro using PKA. The proteins (1 μg for each gel) were then separated by two-dimensional electrophoresis and transferred to Immobilon. Non-phosphorylated HSP20 is shown in A and C. HSP20 phosphorylated by PKA is shown in B and D. A and B were probed with the phosphorylation state-specific antibody and C and D with the antibody that recognizes all isoforms of HSP20. The * denotes a hyperphosphorylated form of HSP20 that is present only when HSP20 is phosphorylated by PKA in vitro (4). The mobility of molecular weight markers is indicated on the left of each panel and the mobility of isoelectric focusing markers on the top of A.

**Fig. 4. Phosphopeptide mapping of isoforms 3, 4, and 8 of HSP20.** Chromatograms from the tryptic digests of HSP20 phosphorylated with PKA (A), isoform 3 (B), and isoform 8 (C) after in situ phosphorylation with 3-isobutyl-1-methylxanthine and forskolin and isoform 4 after in situ phosphorylation with phorbol dibutyrate (D) reveal the relative mobility of the peptide fragments. The fractions containing the peak amount of radioactivity are indicated in Panel A (Peak 1 and Peak 2). A strip of the two-dimensional gel containing isoforms 3, 4, and 8 of HSP20 (E) was digested with staphylococcal V8 protease, and the phosphopeptides generated are depicted in F. There were peptides unique to isoforms 8 and 4 (arrows).
cyclic nucleotide-dependent vasorelaxation, strips of human aortic smooth muscle were labeled with \([^{32}P]\)orthophosphate and treated with IBMX (1 mM) and forskolin (10 μM). The strips were separated by two-dimensional electrophoresis and transferred to Immobilon. Autoradiographs were developed (Fig. 9A), and the blots were then probed with the phosphorylation state-specific antibodies (Fig. 9B). Only isoform 3 was phosphorylated after IBMX and forskolin treatment of human aortic smooth muscle.

The Effect of Synthetic Peptides on Contractile Responses in Permeabilized Bovine Carotid Artery Smooth Muscle—To determine the effect of phosphorylation of HSP20 on smooth muscle physiology, strips of bovine carotid artery smooth muscle were transiently permeabilized and synthetic peptides introduced. The synthetic peptide, WLRRAAPLPGLK, in which Ser16 was phosphorylated, significantly attenuated both KCl (110 mM)- and serotonin (5HT, 1 μM)-induced contractions (Fig. 10). In addition, the synthetic peptide, WLRRAPLPGLK, in
HSP20 Is Phosphorylated on Serine 16

**Fig. 10.** The effect of synthetic peptides on contractile responses of transiently permeabilized vascular smooth muscles. Strips of bovine carotid artery smooth muscles were transiently permeabilized and incubated in the presence of synthetic peptides. The strips were then treated with high potassium (110 mM, KCl), re-equilibrated in bicarbonate buffer, and treated again with serotonin (5HT, 1 μM). A representative tracing of the responses after incubation with the synthetic peptide, WLRRASPLPGLK (in which Ser16 was phosphorylated, dotted line), WLRRASPGLPK (in which Ser16 was replaced with an alanine, dashed line), or with WLRRASPLGPK (in which Ser16 was not phosphorylated, solid line) is depicted in A. Aggregate data, normalized to stress (where stress (10^5 N/m^2) was calculated as force (gms) * 0.0987/area, where area = wet weight (mg)/length (mm) at F_max/V1.055) are depicted in B for KCl responses and C for serotonin responses; lane 1 is the non-phosphorylated peptide; lane 2 is the phosphorylated peptide; and lane 3 is the peptide in which Ser16 was replaced with an alanine (n = 5, * p < 0.05 compared with control). PRKALWGLRPLA, a peptide containing a random distribution of the amino acids, also had no effect on KCl- (110 mM)- or serotonin (5HT, 1 μM)-induced contractions (p > 0.05 compared with control, data not shown).

which Ser16 was replaced with an alanine, thus rendering the peptide “nonphosphorylatable” augmented both KCl (110 mM)- and serotonin (5HT, 1 μM)-induced contractions (Fig. 10). The synthetic peptides, WLRRASPLGPK, in which Ser16 was not phosphorylated, and PRKALWLGRPLA, a peptide containing a random distribution of the amino acids, had no effect on KCl (110 mM)- or serotonin (5HT, 1 μM)-induced contractions (p > 0.05 compared with control, data not shown).

To determine if the phosphorylation state of the peptides could be modified in vitro, the WLRRASPLGPK peptide was phosphorylated by the catalytic subunit of PKA. No increase in phosphorylation of the WLRRASPLGPK peptide (2518 ± 214 base line versus 3379 ± 315, n = 3, p > 0.05) was observed. However, a larger peptide, EIPVPVQPSWLRASPLGPKL, was phosphorylated in vitro by PKA (1258 ± 153 versus 4580 ± 408, n = 3, p < 0.05).

**DISCUSSION**

The present experiments demonstrate that the small heat shock-related protein, HSP20 is phosphorylated on Ser16 during cAMP-dependent vasorelaxation (Fig. 1 and Fig. 2). This serine is contained in a region with a consensus sequence for both PKA and PKG (RRAS). There are three phosphorylated isoforms of HSP20 in bovine carotid artery. Isoforms 3 and 8 are phosphorylated on Ser16 during cAMP-dependent vasorelaxation. Antibodies generated against a peptide containing the Ser16 phosphorylation site were both sensitive and specific for phosphorylated HSP20 (Figs. 5–7). These antibodies recognized isoforms 3 and 8 but did not recognize isoform 4 (Fig. 8). Only one phosphorylated isoform (isoform 3) was recognized in human vascular tissue with the phosphorylation state-specific antibody (Fig. 9). In addition, another small heat shock protein in muscle, MKBP, is not phosphorylated when muscles are stimulated with PDBu or with IBMX/forskolin (Fig. 8). Although this protein has considerable sequence homology with HSP20, it does not contain the RRAS16 site (19). Taken together, these data suggest that there are species differences in HSP20 isoform expression and that Ser16 is the physiologically relevant phosphorylation site for cAMP-dependent vasorelaxation.

The isoforms that are phosphorylated after activation of adenylate cyclase with forskolin or guanylate cyclase with sodium nitroprusside stimulation have similar mobilities on two-dimensional gels (4). The peptide maps after proteolytic digestion of the two phosphorylated isoforms of HSP20 with S. aureus V8 protease are similar (4). The phosphorylation of HSP20 by PKG in vitro resulted in a similar mobility of the phosphorylated peptide on the SMART system as the phosphorylation of HSP20 by PKA in vitro (Fig. 1). Finally, the phosphorylation state-specific antibodies recognize the same phosphorylated isoforms in vessels treated with sodium nitroprusside as with forskolin (Fig. 7). These data suggest that HSP20 is phosphorylated on Ser16 after activation of cGMP-dependent signaling pathways.

Whereas there are three phosphorylated isoforms of HSP20 in bovine carotid artery smooth muscles, two of the isoforms, 4 and 8, have peptide maps that differ from that of isoform 3 (Fig. 4) suggesting that they may represent proteins that contain different amino acid sequences. The simplest explanation of our present results is that isoform 4 is phosphorylated on a carboxyl-terminal site when agonists induce contraction, and then Ser16 is phosphorylated when PKA is activated. This leads to a shift from isoform 4 to isoform 8.

Using the identified phosphorylation site on the HSP20 mol-
HSP20 Is Phosphorylated on Serine 16

The small heat shock proteins (15–30 kDa), B-crystallin, aA-crystallin, HSP20, HSP27, and the MKBP all share considerable sequence homology (approximately 50%) (19). HSP20 and HSP27 also have been shown to be an actin-binding protein, and are not known. However, increases in the phosphorylation of HSP27 have been associated with vascular smooth muscle contraction. This peptide may be inhibiting a HSP20 phosphatase or the peptide may act on the same target as the HSP20 protein. The introduction of a peptide in which the phosphorylated serine was replaced with an alanine enhanced contractile responses (Fig. 10). This peptide may inhibit the phosphorylation of endogenous HSP20 or inhibit the effects of the HSP20 molecule. Peptides that were not phosphorylated or contained a scrambled sequence had no effect on contractile responses. The synthetic peptide could not be phosphorylated by PKA in vitro, suggesting that contractile responses were not altered by phosphorylation of the peptides in the strips of muscle. These data supply direct but incomplete evidence that the phosphorylation of HSP20 may be a critical event in the relaxation of tonic vascular smooth muscle.

Heat shock proteins are a group of proteins whose synthesis is induced by heat or other stressors. These proteins are divided into several groups based on molecular weights. The small heat shock proteins (15–30 kDa), B-crystallin, aA-crystallin, HSP20, HSP27, and the MKBP all share considerable sequence homology (approximately 50%) (19). HSP20 and HSP27 are highly expressed in muscle cells (7), and both exist in phosphorylated and non-phosphorylated forms (4, 20). The specific physiologic functions of the small heat shock proteins are not known. However, increases in the phosphorylation of HSP27 have been associated with vascular smooth muscle contraction (21, 22) and increases in the phosphorylation of HSP20 with vascular smooth muscle relaxation (4). HSP27 has also been implicated in stabilizing the actin cytoskeleton (23). HSP20 has also been shown to be an actin-binding protein, and the association of HSP20 with actin in vitro is dependent on the phosphorylation state of HSP20 (24). Thus, the small heat shock proteins may be late phase signaling molecules that modulate smooth muscle contractile responses via a direct interaction with specific cytoskeletal and/or contractile elements.

In sum, these data suggest that the cyclic nucleotide-dependent vasorelaxation is associated with increases in the phosphorylation of HSP20 at Ser16. Phosphorylation of HSP20 at Ser16 is not only associated with cyclic nucleotide-dependent vasorelaxation but also inhibits agonist-induced contractile responses.

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