Cyclic Nucleotide-dependent Vasorelaxation Is Associated with the Phosphorylation of a Small Heat Shock-related Protein*

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Phosphorylation of a Small Heat Shock-related Protein*

Activation of cyclic nucleotide-dependent signaling pathways leads to the relaxation of various smooth muscles. One of the major phosphorylation events associated with cyclic nucleotide-dependent vasorelaxation in bovine trachealis and carotid artery smooth muscle is the phosphorylation of two 20-kDa phosphoproteins with pI values of 5.7 and 5.9 (previously designated pp8 and pp3, respectively). The present studies sought to determine the identities of pp3 and pp8 in vascular smooth muscle. The phosphopeptide maps for the pp8 and pp3 proteins were similar. Preparative two-dimensional gel electrophoresis and amino acid sequencing of a peptide fragment of the pp3 protein revealed a sequence identical to a 20-kDa heat shock-related protein (HSP20) previously purified from skeletal muscle. Western blot and immunoprecipitation analysis with anti-HSP20 antibodies demonstrated that the pp3 and pp8 proteins are phosphorylated forms of HSP20. In addition, HSP20 could be phosphorylated in vitro by both cAMP-dependent protein kinase and cGMP-dependent protein kinase. These data suggest that the phosphorylation of the heat shock-related protein HSP20 is associated with cyclic nucleotide-dependent relaxation of vascular smooth muscle.

EXPERIMENTAL PROCEDURES

Materials—The catalytic subunit of PKA and endopeptidase Lys-C were purchased from Promega (Madison, WI). The [14C]orthophosphate and [γ-32P]ATP were from Amersham Corp. The cAMP-dependent protein kinase inhibitor peptide was from Peninsula Labs (Belmont, CA). Serotonin and protein A-Sepharose beads were from Sigma. Forskolin, leupeptin, and aprotinin were from Calbiochem. Electrophoresis reagents and the DC protein assay kit were from Bio-Rad. Rabbit anti-HSP20 antibody was produced against purified HSP20 as described...
Phosphorylation of HSP20 during Vasorelaxation

Preparation of Vascular Smooth Muscle Strips—Intact bovine carotid arteries were obtained from an abattoir. The adventitia was dissected free, and the endothelial layer was gently denuded. The arteries were opened longitudinally, and thin transverse strips were cut. The contractile viability of the vessels was confirmed by parallel muscle-bath experiments as described previously (17).

Whole Cell Phosphorylation—Strips of bovine carotid artery smooth muscle were quillipped in a bicarbonate buffer (120 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO4, 1.0 mM Na2HPO4, 10 mM glucose, 1.5 mM CaCl2, and 25 mM NaHCO3) bubbled with 95% O2, 5% CO2 for 1 h at 37°C. The strips were then rinsed and incubated in a low phosphate buffer consisting of 10 mM Heps, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 1.0 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, and 0.3 mM Na2HPO4 for 15–30 min. 250 μCi/ml [32P]orthophosphate was added 3 h before the addition of a vasorelaxant (10 μM forskolin or 10 μM sodium nitroprusside). The incubation was terminated by immersing the muscle strips in a dry ice/acetone slurry and then crushing the tissue with mortar and pestle under liquid N2. The powder was resuspended in homogenization buffer (20 mM Heps, pH 7.4, 20 mM sucrose, 100 mM NaF, 15 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1.3% SDS) and boiled for 5 min. Protein concentrations were normalized using the Bio-Rad DC protein assay kit.

Two-dimensional Gel Electrophoresis—Two-dimensional electrophoresis was performed using vertical slab isoelectric focusing gels with the modification described by Hochstrasser et al. (18). Briefly, the proteins in the samples were acetic-acid-precipitated and reconstituted in 9 μL urea and 2% CHAPS. The samples were protein-normalized, and 100 μg of protein was adjusted to a final concentration of 9 μL urea, 2% CHAPS, 100 mM dithiothreitol, 15% glycerol, and 5% Ampholine (5 parts pH 6–8, 3 parts pH 5–7, 2 parts pH 3–10). The first dimensions were focused for 10,000 Vh and then run on a 12% SDS-PAGE second dimension (19). The gels were stained with Coomassie Brilliant Blue, and the dried gels were exposed to Kodak XAR-5 film.

For peptide sequencing, the second dimension gels were transferred to Immobilon-P (Millipore, Bedford, MA) and stained with Ponceau Red to visualize the spots for isolation and sequencing. Eight to ten gels were transferred, and the pp3 protein was submitted to the Microchemical Facility, Winship Cancer Center, Emory University for amino acid sequencing. The protein was digested using endoproteinase Lys-C and separated by reversed phase microbore HPLC, and selected fragments were sequenced.

Second dimension PAGE gels were transferred to Immobilon-P (Millipore) and blocked with 5% milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 1 h. The blots were incubated with rabbit anti-HSP20 antibody (1:20,000), rabbit anti-α-crystallin (1:2,000), or mouse anti-HSP27 (1:4,000) for 3 h at 4°C. The blots were then washed in TBS, 0.5% Tween 20 (3 washes of 5 min each). Immunoreactive spots were identified using horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-mouse for 1 h at 25°C, and after washing (8 washes of TBS, 0.5% Tween 20, 5 min each), Western blot chemiluminescence reagent was applied (DuPont NEN), and the blots were exposed to Kodak XAR-5 film.

Peptide Mapping—Peptide mapping was performed according to the method of Cleveland et al. (20). The spots corresponding to the 20-kDa proteins pp3 and pp8 were cut from two-dimensional gels and rehydrated with 125 mM Tris, pH 6.8, 0.1% SDS, for 1 h. The rehydrated gel pieces were placed in the wells of a 15% SDS-PAGE gel and overlaid with 10 μg of Staphylococcus aureus V8 protease in 125 mM Tris HCl, pH 6.8, 0.1% SDS, and 15% glycerol. The gel was run at 150 V until the dye front reached the end of the gel. The gels were dehydrated in graded methanol (to 100% methanol), dried, and exposed to Kodak XAR-5 film.

Immunoprecipitation—Strips of bovine carotid artery smooth muscle were homogenized in TBS (0.5 g of tissue/ml of buffer), and then the samples were centrifuged at 10,000 × g for 15 min. The soluble proteins were then diluted 10-fold with TBS. The anti-HSP20 antiserum was added to the supernatants (1:100 dilution). The samples were shaken gently for 14 h at 4°C. Protein A-Sepharose beads (0.1 vol/total sample) were added to the samples. The complexes were incubated for an additional 3 h at 4°C. The beads were washed 3 times with TBS, 0.5% Tween 20. A final wash of 10 mM Tris, pH 7.4, was then done. The immunoprecipitated samples were phosphorylated in vitro (see below) or reconstituted in 9 μL urea, 2% CHAPS, 100 mM dithiothreitol, 15% glycerol, and 5% Ampholine (5 parts pH 6–8, 3 parts pH 5–7, 2 parts pH 3–10) and separated by two-dimensional mini-gel electrophoresis.

Identification of 20-kDa Phosphoproteins—To identify the Phosphorylation of HSP20 in Intact Strips of Bovine Carotid Artery Smooth Muscle—Treatment of strips of bovine carotid artery smooth muscle with either the adenylate cyclase activator forskolin (10 μM) or the guanylate cyclase activator sodium nitroprusside (10 μM) resulted in a significant increase in the phosphorylation of two 20-kDa phosphoproteins with pI values of 5.7 and 5.9 (previously designated pp8 and pp3, respectively (Fig. 1)). Sodium nitroprusside elicited a 2.7-fold increase in pp8 phosphorylation and a 1.8-fold increase in pp3 phosphorylation (Table I). Forskolin stimulated a 5.2-fold increase in pp8 phosphorylation and a 3.0-fold increase in pp3 phosphorylation (Table I). Also, as described previously (14) the phosphorylation of a third 20-kDa protein with a pI of 6.0 (designated pp4) decreased following treatment with either forskolin or sodium nitroprusside (Fig. 1). The increases in the phosphorylation of pp3 and pp8 are the major phosphorylation changes observed during cyclic nucleotide-dependent vasorelaxation of carotid artery smooth muscle as determined with whole cell phosphorylation and two-dimensional gel electrophoresis (14).

To assess the relationship of proteins pp3 and pp8, we performed S. aureus V8 limited proteolysis (20) of pp3 and pp8 phosphorylated in response to either forskolin or sodium nitroprusside. Digests of the two 20-kDa proteins gave similar phosphopeptide maps (Fig. 2). These data suggested that the pp3 and pp8 proteins are structurally related and are phosphorylated within closely related peptide sequences in response to both forskolin and sodium nitroprusside.

Identification of 20-kDa Phosphoproteins—To identify the
TABLE I

Effects of sodium nitroprusside and forskolin on in vivo phosphorylation of pp8 and pp3 in intact bovine carotid artery smooth muscle strips

|          | pp8       | pp3       | Reference B |
|----------|-----------|-----------|-------------|
| Control  | 669 ± 147 | 1985 ± 170| 791 ± 100   |
| Nitroprusside | 1812 ± 300* | 3657 ± 229* | 826 ± 147   |
| Forskolin | 3494 ± 259* | 5990 ± 205* | 755 ± 137   |

*p < 0.05 compared to respective control.

20-kDa proteins, the pp3 phosphoprotein was isolated from preparative two-dimensional gels. A Lys-C peptide digest was resolved on HPLC, and a single major peptide peak was isolated and submitted for sequencing. Amino acid analysis of the Lys-C peptide fragment revealed a sequence of HFSPEELKVK. This sequence contained an 80% sequence identity with the B-crystallin (HFSPEELKVK) and was completely identical to another protein recently purified from skeletal muscle (HSP20 (16)).

To confirm that HSP20 was phosphorylated during cyclic nucleotide-dependent relaxation, immunoblots with anti-HSP20 antibodies were performed. Intact strips of bovine carotid artery smooth muscle were labeled with [32P]orthophosphate and treated for 10 min with either 10 μM forskolin (F) or 10 mM sodium nitroprusside (S). The spots corresponding to the 20-kDa phosphoproteins pp8 and pp3 were excised from the two-dimensional gels. Proteolytic digests of the proteins were performed according to the method of Cleveland et al. (20), the peptides were separated on a 15% SDS-PAGE gel, and autoradiographs were obtained. The pattern of the phosphorylated peptide fragments from the pp3 protein was similar to the pattern for the pp8 protein. The autoradiograph is representative of three separate experiments.

**FIG. 2. Phosphopeptide maps of the pp3 and pp8 20-kDa proteins.** Strips of bovine carotid artery smooth muscle were prelabeled with [32P]orthophosphate and treated for 10 min with either 10 mM forskolin (F) or 10 mM sodium nitroprusside (S). The spots corresponding to the 20-kDa phosphoproteins pp8 and pp3 were excised from the two-dimensional gels. Proteolytic digests of the proteins were performed according to the method of Cleveland et al. (20), the peptides were separated on a 15% SDS-PAGE gel, and autoradiographs were obtained. The pattern of the phosphorylated peptide fragments from the pp3 protein was similar to the pattern for the pp8 protein. The autoradiograph is representative of three separate experiments.

To confirm that HSP20 was phosphorylated during cyclic nucleotide-dependent relaxation, immunoblots with anti-HSP20 antibodies were performed. Intact strips of bovine carotid artery smooth muscle were labeled with [32P]orthophosphate and then stimulated with 10 μM forskolin for 10 min. The strips were homogenized, and the 10,000 × g supernatant proteins were separated by two-dimensional gel electrophoresis. The proteins were transferred to Immobilon, and an autoradiograph was developed. The membranes were subsequently probed with rabbit anti-HSP20 antibodies. Both pp8 and pp3, which demonstrated increases in phosphorylation with CaMP-dependent vasorelaxation, were immunoreactive with anti-HSP20 antibodies (Fig. 3). A more basic non-phosphorylated protein was also immunoreactive with anti-HSP20 antibody. The non-phosphorylated immunoreactivity comigrated with purified rat HSP20 (data not shown). Immunoreactivity for the non-phosphorylated protein decreased with forskolin stimulation. In addition, the pp4 protein was recognized by the HSP20 antiserum and, similar to pp4 phosphorylation, immunoreactivity decreased with stimulation by forskolin. Antibodies against closely related αB-crystallin and another small heat shock protein (HSP27) did not cross-react with any of the HSP20 immunoreactive proteins (data not shown).

Finally, to confirm the identities of pp3 and pp8 intact strips of bovine carotid arteries were stimulated with 10 μM forskolin for 10 min, and muscle proteins were immunoprecipitated with anti-HSP20 antibodies (Fig. 4). The anti-HSP antibodies immunoprecipitated two proteins with similar molecular masses and isoelectric points to the pp3 and pp8 proteins phosphorylated in response to forskolin stimulation (Fig. 4).

**FIG. 3.** The 20-kDa cyclic nucleotide-dependent phosphoproteins are immunoreactive with anti-HSP20 antibodies. Strips of bovine carotid artery smooth muscle were labeled with [32P]orthophosphate and treated either in the absence (A, C) or presence (B, D) of forskolin (10 μM) for 10 min. Arterial homogenates were separated by two-dimensional gel electrophoresis, and the proteins were transferred to Immobilon. The pH gradient is indicated at the bottom of the gels, and molecular mass markers (kDa) are identified at the left. The autoradiographs of phosphorylated proteins (A, B) reveal phosphorylation of the myosin light chains (m) and the 20-kDa phosphoproteins (arrows). The corresponding anti-HSP20 immunoblots (C, D) demonstrate immunoreactive proteins corresponding to the pp8, pp3, and pp4 phosphoproteins. In addition, the immunoblot also identifies a non-phosphorylated protein immunoreactive with anti-HSP20 (*). These blots are representative of three separate experiments.

**DISCUSSION**

Cyclic nucleotide-dependent vasorelaxation is associated with an increase in the phosphorylation of two 20-kDa phosphoproteins, pp8 and pp3 (14, 15). These two phosphoproteins share partial sequence identity and are immunoreactive with a recently identified small heat shock-related protein (HSP20). They appear to represent phosphorylated forms of the same protein. The phosphorylation of HSP20 increases with cyclic
PKG. HSP20 can also be phosphorylated by PKA and forskolin or sodium nitroprusside. These results indicate that phosphorylation of HSP20 is an important event in cyclic nucleotide-dependent relaxation of vascular smooth muscle.

An additional 20-kDa phosphoprotein (pI 6.0) previously referred to as phosphoprotein 4 (pp4 (14)) was also immunoreactive with anti-HSP20 antibodies. However, the phosphorylation of this protein increases with stimuli that induce contraction of the vascular smooth muscle and decreases with stimuli that induce relaxation (14). Treatment of carotid artery smooth muscle with phorbol esters also elicits an increase in pp4 phosphorylation (14). Thus, pp4 may represent a population of HSP20 that is phosphorylated by protein kinase C or another unidentified kinase. Alternatively, it is possible that pp4 represents a different protein that shares homology with HSP20.

Heat shock proteins represent a family of phylogenetically well conserved proteins whose expression is induced by cellular stress (for review, see Ref. 22). Many HSPs (including HSP20) are also expressed constitutively and thus appear to play a role in normal cellular behavior. αB-crystallin, HSP27, and HSP20 are all members of the low molecular weight HSP family (“small HSPs”). The small HSPs share considerable sequence homology and often copurify in large macromolecular aggregates (16, 23). HSP20 was originally identified as a by-product of the purification of HSP27 (16). While HSP20 is ubiquitously distributed, it is found in much higher levels in skeletal, smooth, and heart muscle (16). The prevalence of HSP20 in muscle tissue supports a role for HSP20 in contractile physiology. Unlike αB-crystallin and HSP27, the amount of HSP20 does not increase after heat shock in rat skeletal muscle (16). However, HSP20 does redistribute from a cytosolic to an insoluble fraction and dissociates from an aggregated form after cellular stress (16). Thus, HSP20 does share some of the functional properties of the other small HSPs.

While the precise functions of the HSPs are not known, many HSPs act as “molecular chaperones” and assist in the assembly, disassembly, stabilization, and internal transport of intracellular proteins. Recent studies suggest that small HSPs are important regulatory components of the actin-based cytoskeleton (24), and phosphorylation of HSP27 has been implicated in regulating the contraction of rectal sphincter smooth muscle (25). Other investigations have suggested that small HSPs interact with intermediate filaments (26), which in turn may play a regulatory role in vascular smooth muscle contraction and relaxation (27).

Although the specific role that the phosphorylation of HSP20 plays in vasorelaxation is not known, the phosphorylation of HSP20 may alter its ability to associate with components of either the actin-myosin contractile domain or with the intermediate filament domain of smooth muscle cells (28, 29). The localization of another small HSP (αB-crystallin) with the Z band of cardiac muscle along with the evidence that the dense bodies are the structural counterpart of the Z band in smooth muscle raises the possibility that small HSPs act at the level of the dense bodies (30). Since the dense bodies are the sites at which both the actin-myosin contractile filament and the intermediate filament domains are anchored, cyclic nucleotide-dependent relaxation may lead to a simultaneous reorganization of each fibrillar domain and release the muscle from the so-called “latch” state (31).

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