Localization, Macromolecular Associations, and Function of the Small Heat Shock–Related Protein HSP20 in Rat Heart

Walter Pipkin, MD; John A. Johnson, PhD; Tony L. Creazzo, PhD; Jarrett Burch, BS; Padmini Komalavilas, PhD; Colleen Brophy, MD

**Background**—The small heat shock proteins HSP20, HSP25, αB-crystallin, and myotonic dystrophy kinase binding protein (MKBP) may regulate dynamic changes in the cytoskeleton. For example, the phosphorylation of HSP20 has been associated with relaxation of vascular smooth muscle. This study examined the function of HSP20 in heart muscle.

**Methods and Results**—Western blotting identified immunoreactive HSP20, αB-crystallin, and MKBP in rat heart homogenates. Subcellular fractionation demonstrated that HSP20, αB-crystallin, and MKBP were predominantly in cytosolic fractions. Chromatography with molecular sieving columns revealed that HSP20 and αB-crystallin were associated in an aggregate of ~200 kDa, and αB-crystallin communoprecipitated with HSP20. Immunofluorescence microscopy demonstrated that the pattern of HSP20, αB-crystallin, and actin staining was predominantly in transverse bands. Treatment with sodium nitroprusside led to increases in the phosphorylation of HSP20, as determined with 2-dimensional immunoblots. Incubation of transiently permeabilized myocytes with phosphopeptide analogues of HSP20 led to an increase in the rate of shortening. The increased shortening rate was associated with an increase in the rate of lengthening and a more rapid decay of the calcium transient.

**Conclusions**—HSP20 is associated with αB-crystallin, possibly at the level of the actin sarcomere. Phosphorylated HSP20 increases myocyte shortening rate through increases in calcium uptake and more rapid lengthening. (*Circulation. 2003; 107:469-476.*)

**Key Words:** myocytes ■ muscle, smooth ■ contractility

Severa] recent studies have demonstrated that nitric oxide (NO) has direct effects on myocardial contractile function. Exogenous NO donors characteristically enhance relaxation without major effects on peak systolic function. This selective relaxant action has been observed in intact hearts and cardiac myocytes, and the effects have been attributed to a direct action of NO on the heart. Increases in cGMP and activation of cGMP-dependent protein kinase have been implicated as the cellular mechanism. The specific protein that is phosphorylated by cGMP-dependent protein kinase that produces the relaxant effect in the heart has not been identified.

We have recently determined that cyclic nucleotide–dependent relaxation of vascular smooth muscle is associated with an increase in the phosphorylation of the small heat shock–related protein HSP20. These results have been independently confirmed by two other laboratories. HSP20 is highly and constitutively expressed in muscle tissues and can be phosphorylated in vitro by cGMP-dependent protein kinase. A more direct role for HSP20 in mediating smooth muscle relaxation has been suggested, as follows: (1) Physiological release of NO, induced by acetylcholine or increased flow, leads to measurable increases in the phosphorylation of HSP20; (2) HSP20 is not phosphorylated in muscles that are refractory to cGMP-dependent relaxation; (3) pretreatment of smooth muscles with cyclic nucleotide activators leads to increases in the phosphorylation of HSP20 and inhibition of contraction responses to agonists; (4) the introduction of phosphopeptide analogues of HSP20 into transiently permeabilized vascular smooth muscles inhibits contraction. Thus, cyclic nucleotide-dependent relaxation of smooth muscle seems to be modulated by increases in the phosphorylation of HSP20.

The family of small heat shock proteins, HSP20, HSP25, αB-crystallin, and myotonic dystrophy kinase binding protein (MKBP), are expressed in muscle tissues and share considerable sequence homology, primarily at the c-terminus. These proteins are often associated in large macromolecular aggregates. Heat shock proteins have been implicated in modulating the cellular response to many stressors (heat,
chemical, osmotic, oxidative, and mechanical) and in modulating protein–protein interactions. An additional recently described function of heat shock proteins is to modulate the activity of kinases. MKBP activates myotonic dystrophy protein kinase, and HSP90 modulates the activity of NO synthase. Three of the small heat shock proteins, HSP25, αB-crystallin, and HSP20, are likely involved in cytoskeletal dynamics. HSP25 (the homologous human protein is referred to as HSP27) is an actin-associated protein and modulates actin filament dynamics.15 HSP25 (the homologous human protein is referred to as HSP27) is an actin-associated protein and modulates actin filament dynamics.15–17 HSP20 is also an actin-associated protein.18,19 HSP25 and αB-crystallin both associate with intermediate filament proteins and modulate the interactions that occur between filaments in cellular networks.20 The small heat shock proteins may function to preserve myofibrillar integrity in response to stress and may serve a physiological function in the dynamic cytoskeletal changes associated with contraction and relaxation.

Methods

Materials

Sodium dodecylsulfate (SDS), glycine, tris-(hydroxymethyl) aminomethane (Tris), and diithiothreitol (DTT) were from Research Organics. Coomassie brilliant blue was from ICN Biomedicals Inc. Recombinant HSP27 was from StressGen. Recombinant HSP20 was produced as previously described.21 Piperazine diacrylamide and other electrophoresis reagents were from BioRad. 3-(3-Cholamidopropyl dimethylammonio)-1-propanesulfonate (CHAPS), ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid (EGTA), ethylene diaminetetraacetic acid (EDTA), polyoxyethylene-sorbitan monolaurate (Tween-20), purified bovine αB-crystallin, sodium nitroprusside, and all other reagent-grade chemicals were from Sigma. Protease inhibitor cocktail (2 μmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 130 μmol/L Bestain, 14 μmol/L trans-epoxy succinyl-L-leucylamido(4-guanidino) butane (E-64), 1 μmol/L leupeptin, and 0.3 μmol/L aprotinin, final concentrations) was from Sigma (0.5 g tissue/1 mL buffer) at 4°C. The homogenate was centrifuged at 3000 g to remove debris and the nuclear pellet. The supernatants were diluted to 5 μg of protein/μL, and 250 μL of supernatant was centrifuged at 10 000g for 10 minutes. The pellet (P1) was resuspended in 125 μL of homogenization buffer and 125 μL of 2× sample buffer (6.25 mmol/L Tris, pH 6.8, 2% SDS, 5% 2-β mercaptoethanol, 10% glycerol, 0.025% bromophenol blue). To 125 μL of the 10 000g supernatant (S1), 125 μL of 2× sample buffer was added. The remaining 125 μL of 10 000g supernatant was centrifuged at 100 000g. The 100 000g pellet (P2) was resuspended in 125 μL of homogenization buffer and 125 μL of 2× sample buffer. A total of 125 μL of 2× sample buffer was added to the 100 000g supernatant (S2). The samples were boiled for 8 minutes after the addition of sample buffer, separated on 15% SDS-PAGE gels, transferred to Immobilon membranes, and probed with anti-actin antibodies (1/2000 dilution), anti-HSP20 antibodies (1/2000 dilution), anti-MKBP antibodies (1/2000 dilution), and anti-rabbit secondary antibodies (1/2000 dilution). Gel Filtration

Gel filtration was performed as previously described.13 In brief, the tissues were homogenized in HEPES buffer (0.5 g tissue/1 mL buffer) at 4°C. The homogenate was centrifuged at 100 000g for 30 minutes at 4°C. 200 μL of supernatant (containing 200 μg total protein) was applied to a Superose-6 HR 10/30 fast protein liquid chromatography column (Pharmacia), eluted with column buffer, and 0.5-mL fractions collected. For calibration, 100 μL of catalase (232 kDa, 5 mg/mL) and BSA (67 kDa, 8 mg/mL) was applied to the column.

Subcellular Fractionation

Tissues were homogenized in homogenization buffer (HEPES buffer, 25 mmol/L HEPES, 150 mmol/L NaCl, 10 mmol/L EDTA, 1 mM L- DTT, 2 mM benzamidine, pH 7.4, and protease inhibitor cocktail [2 μmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 130 μmol/L Bestain, 14 μmol/L trans-epoxy succinyl-L-leucylamido(4-guanidino)butane (E-64), 1 μmol/L leupeptin, and 0.3 μmol/L aprotinin, final concentrations]) from Sigma) (0.5 g tissue/1 mL buffer) at 4°C. The homogenate was centrifuged at 10 000g to remove debris and the nuclear pellet. The supernatants were diluted to 5 μg of protein/μL, and 250 μL of supernatant was centrifuged at 10 000g for 10 minutes. The pellet (P1) was resuspended in 125 μL of homogenization buffer and 125 μL of 2× sample buffer (6.25 mmol/L Tris, pH 6.8, 2% SDS, 5% 2-β mercaptoethanol, 10% glycerol, 0.025% bromophenol blue). To 125 μL of the 10 000g supernatant (S1), 125 μL of 2× sample buffer was added. The remaining 125 μL of 10 000g supernatant was centrifuged at 100 000g. The 100 000g pellet (P2) was resuspended in 125 μL of homogenization buffer and 125 μL of 2× sample buffer. A total of 125 μL of 2× sample buffer was added to the 100 000g supernatant (S2). The samples were boiled for 8 minutes after the addition of sample buffer, separated on 15% SDS-PAGE gels, transferred to Immobilon membranes, and probed with anti-actin antibodies (1/2000 dilution), anti-HSP20 antibodies (1/2000 dilution), anti-MKBP antibodies (1/2000 dilution), and anti-rabbit secondary antibodies (1/2000 dilution). Gel Filtration

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Dot Blotting

One hundred microliters of each fraction from the column was dot-blotted onto nitrocellulose. The blots were fixed with 20% methanol, dried, blocked with PBS, 5% milk, for 1 hour, washed 3 times with PBS, and then probed with anti-HSP20, anti-MKBP, and anti-αB-crystallin antibodies (1/2000 dilution in PBS, 5% milk) for 1 hour. Goat anti-rabbit secondary antibodies (1/2000 dilution) were added to the blots for 1 hour. The blots were then washed 6 times with PBS/0.5% Tween-20. Immunoreactive proteins were visualized as described above with enhanced chemiluminescence, and densitometric analysis was performed with UN-SCAN-IT automated digitizing software (Silk Scientific Corporation).

Immunoprecipitation

Tissue was homogenized in TBS (0.5 g tissue per mL of buffer), and then the samples were centrifuged at 10 000g for 15 minutes. The soluble proteins were then diluted 10-fold with TBS. The anti-HSP20 antisera was added to the supernatants (1:50 dilution). The
samples were shaken gently for 14 hours at 4°C. Protein A-Sepharose beads (1/10 volume) were added, and the samples were incubated for an additional 3 hours at 4°C. The beads were washed 6 times with TBS, 0.5% Tween-20. A final wash of 10 mmol/L Tris pH 7.4 was then done. The proteins were separated on 15% SDS-PAGE gels, transferred to Immobilon, and probed with anti-HSP20, anti-αB-crystallin, or anti-MKBP antibodies as described above.

**Immunofluorescence Microscopy**

Perfusion-fixed ventricular tissue was embedded in paraffin. Five-micron cross-sections were mounted on polylysine slides. The slides were deparaffinized with xylene and graded dilutions of ethanol. Neonatal cardiac myocytes were grown on glass coverslips and fixed with 4% paraformaldehyde. The sections were rinsed in PBS and blocked with donkey serum (Jackson Immunoresearch, West Grove, Pa) for 30 minutes. The slides were then incubated with anti-HSP20 (1/100), anti-αB-crystallin (1/100), anti-sarcromeric actin (1/100), or anti-MKBP (1/100) overnight at 4°C. The slides were then washed with PBS, 4 times (15 minutes each), at room temperature. Mouse and anti-goat Cy3 secondary antibodies (1/100) were used to detect immunoreactive protein. The sections were imaged on a Zeiss Axiophot microscope interfaced with a SPOT camera (Diagnostic Instruments) and a Gateway computer.

**Dimensional Gels**

Tissues were snap-frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. The proteins were solubilized in 100 mmol/L DTT, 6 mol/L urea, 2% CHAPS overnight. Protein 30 μg was loaded onto 12×15-cm slab isofocusing gels consisting of 4% acrylamide, 0.1% piperazone diacrylamide, 9 mol/L urea, 5% ampholines (5 parts 6 to 8, 3 parts 5 to 7, and 2 parts 3 to 10), and 2% CHAPS. The cathode buffer consisted of 20 mmol/L sodium hydroxide and the anode buffer 10 mmol/L phosphoric acid. The proteins were focused for 10 000 volt hours. The gels were fixed in 10% trichloracetic acid and stained overnight with Neuhoff’s Coomassie stain. The lanes of stained proteins were cut from the isoelectric focusing gel and equilibrated in 10 mmol/L Tris (pH 6.8), 3% SDS, 19% ethanol, 4% β-mercaptoethanol, and 0.004% bromophenol blue for 10 minutes. The proteins were then separated on 12% acrylamide SDS gels and transferred to Immobilon 100 mAmp for 12 hours. The blots were probed for HSP20 as described above. The isoelectric focusing gradient was determined with BioRad IEF standards.

**Peptide Synthesis**

The peptides, HSP20 phosphoserine analogue N-WLRRASsometimes APLPGLK (HSP20-PS), a scrambled phosphorylated peptide N-PRKStimesWLALGRLPA (HSP20-SC), and a nonphosphorylated peptide N-WLRRAAAPLPGLK (HSP20-NP), were synthesized on a Procise (Applied Biosystems, Model 492) instrument according to standard protocols. The peptides were purified with high-pressure liquid chromatography, and purity was assured with mass spectrometry, as previously described.

**Permeabilization of Cardiac Myocytes**

Permeabilization of cardiac myocytes was performed as described. Briefly, cells were slowly cooled by sequential 2-minute incubations with room temperature PBS and then with 4°C PBS in an ice bath for 2 minutes. The PBS was discarded, and the cells were incubated with ATP (30 μL of 200 mmol/L ATP, pH 7.4) followed immediately by permeabilization buffer (20 mmol/L HEPES, pH 7.4, 10 mmol/L EGTA, 140 mmol/L KCl, 50 μg/mL saponin, 5 mmol/L oxalic acid dipotassium salt) containing the peptides (10 μmol/L) for 10 minutes in an ice bath. The cells were then washed 4 times on ice with chilled PBS. The cells were then returned to 37°C by incubations with room temperature PBS and 37°C PBS. The original cell media was then added at 37°C.

**Measurement of Cardiac Myocyte Shortening Rate/Lengthening Rate**

The culture dishes containing the myocytes were placed on a Harvard Apparatus temperature-regulation device positioned on the stage of an inverted microscope (Carl Zeiss Inc) and maintained at 37°C with a jacketed water bath. The microscope was outfitted with a digital camera and Video Savant software. To determine the effects of HSP20 peptide analogues on shortening rate, individual cells were monitored before and after permeabilization. Shortening rates were determined every 2 minutes (for a 15-second period) for a total of 10 minutes, as previously described. Images of individual cells were captured at 30 frames/second.

**Calcium Transients**

Ca2+ transients were measured as previously described in detail elsewhere with only minor modifications. For these experiments, myocytes were cultured on 25-mm glass coverslips. After permeabilization (as described above), each coverslip was mounted in a leak-proof circular holding chamber (Medical Systems Inc) and gently washed several times with a Ringer solution containing (in mmol/L) NaCl 142.5, KCl 4.0, MgCl2 1.8, CaCl2 1.8, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid 5.0 (HEPES, pH 7.0), and glucose 5.0. After washing, the myocytes were incubated in 1 mL Ringer solution with 1 μmol/L fura-2 AM for 10 minutes at 37°C in a rotating water bath. The myocytes were subsequently washed several times with dye-free Ringer solution and allowed to stand covered at room temperature for 30 minutes to facilitate deesterification.

Fluorescence measurements in individual rhythmically beating myocytes were carried out at 37°C with the use of a DeltaScan microspectrofluorimeter (Photon Technology International) coupled with an Olympus IX70 microscope equipped with an Olympus U Apo/340×40 oil immersion objective with a numerical aperture of 1.35. The fura-2 transients reported are the ratio of Ca2+ fluorescence transients measured at excitation wavelengths 340 and 380 nm. The myocytes were only illuminated at short intervals, and each sample preparation was used for <1 hour to reduce the possibility of photobleaching or fura-2 leakage.

**Statistical Analysis**

Values are reported as mean±SEM, and n refers to the number of animals examined. The statistical differences between 2 groups were determined by Student’s t test and between multiple groups by 1-way repeated-measures ANOVA with Sigma Stat software (Jandel Scientific). P<0.05 was considered significant.

**Results**

**Specificity of Small HSP Antibodies**

The small heat shock proteins are highly homologous, thus, to determine the specificity of the antibodies, immunoblots of recombinant HSP20, recombinant HSP25, bovine lens αB-crystallin, and homogenized rat heart were probed. The HSP20, αB-crystallin, and MKBP antibodies recognized the corresponding purified or recombinant protein (Figure 1). The anti-HSP20 antibodies recognized a major band at 20 kDa with a smaller band at a slightly lower relative mobility in heart homogenate proteins (Figure 1, HSP20). This additional lower band may represent cross-reactivity with other proteins or may represent HSP20 that has been posttranslationally modified (eg, phosphorylated and nonphosphorylated HSP20). The anti-αB-crystallin antibodies recognized a single band with a relative mobility of 20 kDa in heart homogenates (Figure 1, Crystallin). The immunoreactive band above 20 kDa in the lane containing purified αB-crystallin may represent dimerized αB-crystallin. The anti-
MKBP antibodies recognized a band with a relative mobility of 20 kDa and another band with a higher relative mobility (~35 kDa) in heart homogenates (Figure 1, MKBP). The additional band may represent cross-reactivity with other proteins or dimerized MKBP. There were very low levels of immunoreactive HSP25 detected in adult rat heart with an affinity-purified mouse monoclonal antibody or commercially available antibodies (data not shown). It has been previously reported that HSP25 is highly expressed in neonatal cardiac tissue but is markedly downregulated in adult heart tissues. Only the associations between HSP20, αB-crystallin, and MKBP were examined in subsequent studies.

Subcellular Localization and Macromolecular Associations of the Small HSPs

To determine the intracellular distribution of the small heat shock proteins in cardiac myocytes, subcellular fractionation was performed as described in Methods. HSP20 and MKBP were in the cytosolic fraction in rat heart homogenates (Figure 2, HSP20 and MKBP). αB-crystallin was predominantly in the cytosolic fraction, but there was also a minor component of immunoreactive αB-crystallin in the particulate fraction (Figure 2, crystallin).

The small heat shock proteins have been shown to be associated with each other in macromolecular aggregates, and MKBP and HSP25 were examined in subsequent studies. MKBP antibodies recognized a band with a relative mobility of 20 kDa and another band with a higher relative mobility (~35 kDa) in heart homogenates (Figure 1, MKBP). The additional band may represent cross-reactivity with other proteins or dimerized MKBP. There were very low levels of immunoreactive HSP25 detected in adult rat heart with an affinity-purified mouse monoclonal antibody or commercially available antibodies (data not shown). It has been previously reported that HSP25 is highly expressed in neonatal cardiac tissue but is markedly downregulated in adult heart tissues. Only the associations between HSP20, αB-crystallin, and MKBP were examined in subsequent studies.

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The small heat shock proteins have been shown to be associated with each other in macromolecular aggregates. Fractions from the 10 000g supernatant (S1), 10 000g pellet in (P1), 100 000g supernatant (S2), and 100 000g pellet (P2) were probed with anti-HSP20 antibodies (HSP20, 1:1000 dilution), anti-αB-crystallin antibodies (crystallin, 1:1000 dilution), or anti-MKBP antibodies (MKBP, 1:1000 dilution). The relative mobility of molecular weight standards is indicated on the left of each panel. Arrows depict the relative immunoreactive proteins. This blot is representative of 3 separate experiments.

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Because the small HSPs were predominantly cytosolic proteins, gel filtration was performed on the cytosolic fractions of heart muscle homogenates with superose 6 columns. Fractions from the column were dot-blotted with antibodies against HSP20, αB-crystallin, and MKBP. HSP20 and αB-crystallin were found in similar fractions that eluted from the column after the catalase standard (MW 232, Figure 3A). MKBP eluted from the column in the same fraction as the BSA standard (MW 67 kDa, Figure 3A).

To confirm the associations of the small heat shock protein in rat heart, we performed coimmunoprecipitations. Immunoprecipitation was performed with antibodies against HSP20, the proteins separated and probed with antibodies against HSP20, αB-crystallin, MKBP, and sarcomeric actin. αB-crystallin coimmunoprecipitated with HSP20 (Figure 3B). Neither MKBP nor sarcomeric actin coimmunoprecipitated with HSP20 from rat heart homogenates (data not shown). The secondary antibodies also recognized the light chains of the rabbit polyclonal antibodies used to perform the immunoprecipitations (Figure 3B, 50-kDa band). HSP20, αB-crystallin, and MKBP all have a relative mobility of 20 kDa; thus, they could readily be detected at a relative mobility lower on the gel than the rabbit polyclonal antibodies used in the immunoprecipitations. The antibodies used to probe for sarcomeric actin were mouse monoclonal. Thus, a signal from the immunoprecipitating antibody would not interfere with the actin probe. There was no nonspecific immunoreactive protein when heart homogenates were immunoprecipitated with preimmune rabbit serum (data not shown). The
antibodies used to detect αB-crystallin did not immunoprecipitate αB-crystallin; thus, experiments to immunoprecipitate αB-crystallin and detect immunoreactive HSP20 were not performed.

**Cellular Localization of the Small HSPs With Immunofluorescence Microscopy**

To additionally localize the small HSPs in rat heart, immunofluorescence microscopy was performed. Immunoreactive sarcomeric actin was present in distinct transverse bands (Figure 4A). Immunoreactive αB-crystallin was also present in transverse bands, but the staining was more punctate (Figure 4B). Immunoreactive HSP20 was present in transverse bands, and there was also staining of the cell membranes (Figure 4C). MKBP was not included because of the limited specificity of the MKBP antibodies. Neonatal cardiac myocytes were also stained with anti-actin and anti-HSP20 antibodies, and similar staining patterns were observed (Figures 4D and 4E). There was no specific pattern of immunoreactivity using preimmune serum or no primary antibody (data not shown).

**Activation of Cyclic Nucleotide Signaling Pathways Leads to an Increase in the Phosphorylation of HSP20 in Cardiac Myocytes**

To determine if activation of cyclic nucleotide signaling pathways leads to increases in the phosphorylation of HSP20, two-dimensional immunoblotting was performed. Increases
in the phosphorylation of HSP20 in vascular smooth muscle leads to a shift of HSP20 from a basic to more acidic isoforms. Rat cardiac myocytes were equilibrated in bicarbonate buffer for 1 hour. The myocytes were then treated with buffer alone or with the NO donor sodium nitroprusside (10 μmol/L, 10 minutes). The cells were homogenized and separated by two-dimensional electrophoresis, transferred to Immobilon, and probed with anti-HSP20 antibodies (1:1000 dilution). The relative mobility of molecular weight standards is indicated at the left of each panel, and the mobility of IEF standards is on the top of panel B. The arrows represent immunoreactive HSP20. This blot is representative of 3 separate experiments.

Phosphorylated Peptide Analogues of HSP20 Leads to an Increase in the Rate of Shortening in Cardiac Myocytes

To determine if HSP20 has a direct role in myocyte function, a model of transient permeabilization of cultured myocytes was used. The rate of shortening and lengthening in unloaded isolated cells was used as a measure of contraction. Introduction of the phosphorylated peptide analogue of HSP20 resulted in a significant increase in the rate of shortening (37.6±2.4%, Figure 6A). Similar increases in the rate of shortening occurred after treatment with sodium nitroprusside (10 μmol/L, 33.5±5.3%). In experiments where the myocytes were permeabilized in the absence of peptide (sham, 1.1±0.5% increase, Figure 6A) or in the presence of scrambled phosphorylated peptide (5.9±1.9% increase, Figure 6A) or nonphosphorylated peptide (5.5±2.4%, Figure 6A), there was no significant change in the shortening rate.

Figure 5. HSP20 is phosphorylated by activation of cyclic nucleotide signaling pathways in neonatal ventricular myocytes. Cardiac myocytes were treated with PBS (A) or sodium nitroprusside (10 μmol/L for 10 minutes) (B). The cells were then homogenized, solubilized, and separated by 2-dimensional electrophoresis. The proteins were then transferred to Immobilon and probed with anti-HSP20 antibodies (1:1000 dilution). The relative mobility of molecular weight standards is indicated at the left of each panel, and the mobility of IEF standards is on the top of panel B. The arrows represent immunoreactive HSP20. This blot is representative of 3 separate experiments.

Figure 6. The effect of phosphopeptide analogues of HSP20 on myocyte function. The basal rate of shortening and lengthening of unloaded isolated cardiac myocytes was determined by computer-assisted digital microscopy imaging techniques. The cells were then transiently permeabilized and incubated with no peptide (sham, SM); N-PRKSphosLWALGRPLA (SC, a scrambled phosphorylated peptide); N-WLRRAAAPLPGLK (NP, a nonphosphorylated peptide); or N-WLRASphosAPLPGLK (PS, the phosphoHSP20 peptide analogue). After permeabilization, the cells were placed back on the microscope stage and the rate of shortening and lengthening was again determined. The data represent the percent increase in rate of change over basal rate (percent increase in rate, A; n=25 cells per group from 5 different experiments; **P<0.05). With the use of digital camera and Video Savant software, images of individual cells were captured at 30 frames per second before (PRE) and after (POST) permeabilization and introduction of the PS peptide analogue. The time from contraction (maximal shortening) to relaxation (maximal lengthening) was measured (relaxation time in seconds; B, n=16 cells from 5 different experiments; **P<0.05).
were collected for 20 to 25 seconds and used only if the basal
calculated ratio of 0.242 ± 0.05 for the myocytes permeabilized in the absence of any phos-
ephopeptide (0.242 ± 0.05). These data indicated that permeabilization with phosphopeptides resulted in a nonspecific decrease in the magnitude of the
transients. The diastolic Ca\textsuperscript{2+} level was not significantly different in any of the 3 groups.

**Discussion**

The small heat shock proteins share considerable homology and are often found in association with other small heat shock proteins. Despite the sequence homology, the antibodies used in these studies were specific for the individual heat shock protein (Figure 1). The small HSPs HSP20, αB-crystallin, and MKBP were predominantly cytosolic proteins, although some αB-crystallin was in a particulate fraction (Figure 2).

Molecular sieving columns suggested that αB-crystallin and HSP20 coexist in a macromolecular aggregate of ~200 kDa (Figure 3A). MKBP was in a much smaller aggregate (60 to 70 kDa). This association was confirmed in that αB-crystallin coimmunoprecipitated with HSP20 (Figure 3B). Immunofluorescence microscopy demonstrated that αB-crystallin and HSP20 had a staining pattern of distinct transverse bands similar to the pattern observed after staining for sarcromeric actin (Figure 4). Thus, both biochemical analyses and immunofluorescence microscopy suggest that HSP20 and α B-crystallin are associated proteins in adult rat hearts. Other studies have suggested that HSP20 is an actin-associated protein in vascular smooth muscle.\textsubscript{18,19} The localization of the small heat shock proteins to specific cytoskeletal domains suggests a role for these proteins in regulating cytoskeletal dynamics.

HSP25, HSP20, and αB-crystallin can be posttranslationally modified by phosphorylation. HSP20 is phosphorylated by cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG).\textsuperscript{1} Increases in the phosphorylation of HSP20 are associated with relaxation of vascular and skeletal muscle.\textsuperscript{3,5,7} In the present investigation, we demonstrate that treatment of neonatal ventricular tissue with sodium nitroprusside leads to increases in the rate of shortening and increases in the phosphorylation of HSP20 (Figure 5). The addition of phosphopeptide analogues of HSP20 into transiently permeabilized cardiac myocytes led to increases in the rate of shortening of the myocytes (Figure 6). These results are consistent with previously reported findings demonstrating that the NO-cGMP pathway increases beat rate by stimulating the hyperpolarization-activated pacemaker current, \(I_{h}\).\textsuperscript{27} The present results suggest that the mechanism of NO stimulation of heart rate via \(I_{h}\) involves phosphorylation of HSP20 and that phosphorylated HSP20 may have a direct effect on \(I_{h}\) channels. The increased rate of shortening was also related to increases in the rate of relaxation of the myocytes (Figure 6). Although there was no change in the magnitude of the Ca\textsuperscript{2+} transient after treatment with the phosphopeptide analogues of HSP20, there was an increase in the rate of decline of the Ca\textsuperscript{2+} transient (Figure 7). This suggests that the phosphopeptide analogue of HSP20 facilitates the increased rate by stimulating a more rapid uptake of Ca\textsuperscript{2+} by the sarcoplasmic reticulum. Taken together, these data suggest that HSP20 may have a direct role in modulating the lusitropic actions of NO and NO donors.

In summary, HSP20 is biochemically associated with αB-crystallin and localizes to distinct transverse bands in a similar pattern as αB-crystallin and sarcomeric actin. The
possible association of HSP20 and αB-crystallin with actin suggests that these two small heat shock proteins may be involved in modulating cytoskeletal or contractile dynamics of cardiac myocytes. Specifically, our results show that the addition of phosphorylated peptide analogues of HSP20 leads to increases in the rate of shortening, which was associated with a more rapid uptake of Ca$^{2+}$ in cardiac myocytes. This suggests that HSP20 may have a physiological role in cardiac myocyte function.

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