Signal Transduction in Smooth Muscle
Selected Contribution: HSP20 phosphorylation in nitroglycerin- and forskolin-induced sustained reductions in swine carotid media tone

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Rembold, Christopher M., Matthew O’Connor, Michael Clarkson, Robert L. Wardle, and Richard A. Murphy. Selected Contribution: HSP20 phosphorylation in nitroglycerin- and forskolin-induced sustained reductions in swine carotid media tone. J Appl Physiol 91: 1460–1466, 2001.—Cyclic nucleotide-induced relaxation of maximally activated arterial smooth muscle has two phases. 1) The initial relaxation transient is typically characterized by a rapid reduction in force associated with brief reductions in myoplasmic Ca^{2+} concentration ([Ca^{2+}]_i) and myosin regulatory light chain (MRLC) phosphorylation on serine (Ser)-19 (Ser19). 2) The sustained inhibitory response is typically associated with Ser16 phosphorylation of heat shock protein 20 (HSP20) without sustained reductions in [Ca^{2+}]_i or MRLC phosphorylation. We investigated whether the extent of Ser16-HSP20 phosphorylation quantitatively correlated with the sustained inhibitory response. With addition of nitroglycerin to histamine-stimulated swine carotid media, the initial relaxation transient was associated with a decrease in MRLC phosphorylation without an increase in Ser16-HSP20 phosphorylation. During the sustained phase of nitroglycerin-induced relaxation and during force redevelopment induced by washout of nitroglycerin in the continued presence of histamine, the level of Ser16-HSP20 phosphorylation, but not MRLC phosphorylation, correlated with inhibition of force. Forskolin, which increases cAMP concentration, also induced a sustained inhibitory response that was associated with increases in Ser16-HSP20 phosphorylation with reductions in MRLC phosphorylation levels. Forskolin increased Ser16-HSP20 phosphorylation to a greater extent and inhibited force more completely than that observed with nitroglycerin. Increases in Ser16-HSP20 phosphorylation correlated with the degree of force inhibition regardless of whether the relaxation was induced by nitroglycerin or forskolin. These data are consistent with the hypothesis that Ser16-HSP20 phosphorylation may be a cyclic nucleotide-dependent, yet MRLC phosphorylation-independent, inhibitor of smooth muscle contractile force.

CONTRACTION OF SMOOTH MUSCLE in response to excitatory neurotransmitters, hormones, or depolarization follows a widely recognized pathway: increased myoplasmic Ca^{2+} concentration ([Ca^{2+}]_i), formation of a Ca^{2+}-calmodulin complex, activation of myosin light chain kinase, and phosphorylation of myosin regulatory light chains (MRLC) on serine (Ser)-19 (Ser19) (12). Phosphorylation of MRLC enables cross-bridge attachment to the thin filament, thereby allowing cross-bridge cycling and force generation (10). In many cases, smooth muscle relaxation proceeds via a reversal of this contraction process: reduction of [Ca^{2+}]_i, inactivation of myosin light chain kinase, and dephosphorylation of MRLC (9, 22, 29).

Smooth muscle relaxation induced by increases in cAMP or cGMP appears to be more complex. In submaximally contracted swine carotid media, addition of forskolin (to increase cAMP concentration) (20) or nitroglycerin (to increase cGMP concentration) (18) induced relaxations that were associated with both initial and sustained reductions in [Ca^{2+}]_i and MRLC phosphorylation without a significant alteration in the Ca^{2+} sensitivity of MRLC phosphorylation. These findings suggest that cAMP or cGMP induced relaxation of
submaximally stimulated tissues primarily by reductions in [Ca\(^{2+}\)]i-dependent MRLC phosphorylation.

When maximally contracted swine carotid media was treated with nitroglycerin, there was a rapid relaxation transient that was associated with a decrease in [Ca\(^{2+}\)]i and MRLC phosphorylation (18). However, low force persisted despite a return of [Ca\(^{2+}\)]i and MRLC phosphorylation to high levels indistinguishable from the levels observed in maximally contracted tissue (18). This phenomenon has been termed “relaxation without MRLC dephosphorylation,” “the sustained inhibitory response,” and “uncoupling of force from MRLC phosphorylation.” It is best characterized as a rightward shift in the MRLC phosphorylation-force relation (18, 19, 24). This response is observed with activators of guanylyl cyclase, such as nitric oxide, and with phosphodiesterase inhibitors that increase intracellular cGMP concentration (6). Other treatments that induce relaxation without MRLC dephosphorylation include okadaic acid (27), some Ca\(^{2+}\) channel blockers (14), high extracellular Mg\(^{2+}\) concentration (7), and combinations of excitatory and inhibitory stimuli (2). Although this phenomenon was studied primarily in vascular smooth muscle, it is most prominent in tissues such as the corpus cavernosum, where inhibitory innervation is a key physiological control mechanism (6).

One potential explanation for sustained low tone without MRLC dephosphorylation could be phosphorylation of MRLC on an amino acid residue that does not activate cross-bridge cycling. However, we found that nitroglycerin phosphorylated MRLC entirely on Ser19 during nitroglycerin-induced relaxation (18) and that nitroglycerin phosphorylated MRLC to high levels indistinguishable from the levels observed in maximally contracted tissue (18). This result indicates that the phosphorylation of MRLC at sites other than Ser19 cannot explain relaxation without proportional MRLC dephosphorylation. Indeed, such data clearly point to the existence of mechanisms that can block force generation by phosphorylated cross bridges in smooth muscle.

Recently, the sustained phase of cAMP- and cGMP-dependent relaxation was associated with phosphorylation of heat shock protein 20 (HSP20) on Ser\(^{16}\) during nitroglycerin-induced relaxation (18) and high extracellular Mg\(^{2+}\) concentration-induced relaxation (7). Ser\(^{16}\) is the phosphorylation site that regulates myosin ATPase activity (21). This result indicates that the phosphorylation of MRLC at sites other than Ser\(^{16}\) cannot explain relaxation without proportional MRLC dephosphorylation. Indeed, such data clearly point to the existence of mechanisms that can block force generation by phosphorylated cross bridges in smooth muscle.

**Fig. 1. Specificity of the anti-heat shock protein 20 (HSP20) antibody.** Homogenates of a swine carotid media (stimulated with 10 \(\mu\)M histamine for 10 min and then relaxed by addition of 10 \(\mu\)M nitroglycerin for 20 min) were separated by isoelectric focusing and blotted to nitrocellulose paper. A: incubated in 1:10,000 anti-HSP20 antibody that was preincubated with 10 \(\mu\)g of recombinant human HSP20 (equivalent to 0.6 \(\mu\)M HSP20) for 30 min at 22°C and then developed with ECL. B: incubated in 1:10,000 anti-HSP20 antibody that was preincubated with 10 \(\mu\)g of recombinant human HSP20 (equivalent to 0.6 \(\mu\)M HSP20) for 30 min at 22°C and then developed with ECL. B shows lack of HSP20 immunoreactivity when antibody was preincubated with HSP20 antigen, demonstrating antibody specificity for HSP20.
antibody with recombinant HSP20 abolished immunostaining of a blot containing swine carotid HSP20.

Measurement of HSP20 and MRLC phosphorylation. Swine carotid arteries were pharmacologically treated and frozen in an acetone-dry ice slurry (25). After air drying, the tissues were homogenized in a buffer containing 1% SDS, 10% glycerol, and 20 mM dithiothreitol (20 mg wet wt/ml buffer). Full-strength, half-strength, and quarter-strength dilutions of samples were then separated on one-dimensional isoelectric focusing gels [ampholytes were a 50:50 mixture of isoelectric point (pl) 5–8 and pl 4–6.5 for HSP20 and a 50:50 mixture of pl 4.5–5.4 and pl 4.0–6.5 for MRLC], blotted to nitrocellulose, immunostained with our rabbit polyclonal anti-HSP20 antibody (1:5,000) or rabbit polyclonal anti-MRLC antibody (1:4,000 in 1% bovine serum albumin and 0.01% sodium azide), and detected with enhanced chemiluminescence (26). The dilutions ensured that the enhanced chemiluminescence detection system was in the linear range (26). Immunoblots were scanned on a Hewlett Packard flatbed scanner and quantitated with UNSCANIT software.

MRLC phosphorylation is reported as suprabasal phosphorylation. Basal MRLC phosphorylation was 0.20 ± 0.05 mol P/mol MRLC (mean ± SE data from Fig. 4), a value higher than we previously reported with two-dimensional gel electrophoresis (25). The increase in our estimates of basal phosphorylation appears to depend on the measurement methodology.

HSP20 has at least two phosphorylation sites. In the unstimulated swine carotid, our laboratory (24) found that over 90% of immunoreactive HSP20 was present in a band at pl 6.0 and that nitroglycerin-induced relaxation was associated with migration of some of the HSP20 immunostaining to a band at pl 5.7 (pl identification as in (4)). Our laboratory interpreted this pl shift as a phosphorylation reaction. A less prominent band at pl 6.3 was also seen (Fig. 1). It was also previously reported (24) that the HSP20 band at pl 5.7 was phosphorylated at Ser16 based on mass spectroscopy sequencing (sequencing did not rule out additional phosphorylation at another site).

Unfortunately, this was an oversimplification. Beall et al. (3) proposed that protein kinase A and protein kinase G phosphorylate HSP20 on Ser16 and protein kinase C phosphorylates HSP20 at another site. Incubation of swine carotid homogenates for 30 min with calf alkaline phosphatase collapsed all immunoreactive HSP20 to a single basic band at pl 6.3. This isoelectric focusing gels (ampholytes were a 50:50 mixture of pl 4.5–5.4 and pl 4.0–6.5 for MRLC), blotted to nitrocellulose, immunostained with our rabbit polyclonal anti-HSP20 antibody (1:5,000), and developed with amplified Opti-4CN (Bio-Rad). Treatment with calf alkaline phosphatase collapsed immunostaining to a single basic band at pl 6.3.

RESULTS

Time course of nitroglycerin-induced relaxation and HSP20 phosphorylation. If Ser16-HSP20 phosphorylation regulates the sustained inhibitory phase of relaxation, then the time course of Ser16-HSP20 phosphorylation should correlate with the sustained inhibitory phase. Maximal stimulation of swine carotid media with 10 μM histamine increased suprabasal MRLC phosphorylation and contractile stress without significantly increasing Ser16-HSP20 phosphorylation (Fig. 3). Force and MRLC phosphorylation decreased rapidly after addition of 10 μM nitroglycerin. One minute after addition of nitroglycerin, Ser16-HSP20 phosphorylation was not significantly increased. These data demonstrate that Ser16-HSP20 phosphorylation cannot account for the initial nitroglycerin-induced relaxation transient. Previous work in our laboratory (18) demonstrated that the initial phase of nitroglycerin-induced relaxation was associated with significant reductions in [Ca2+]i and MRLC phosphorylation. Therefore, the initial phase of relaxation appears to be caused by a reduction in [Ca2+]i-dependent MRLC phosphorylation.

Contrastive force was still declining 2 min after addition of nitroglycerin. Ser16-HSP20 phosphorylation was significantly increased, and suprabasal MRLC phosphorylation had reversed its decline and was increasing. The relaxation was complete 15 min after addition of nitroglycerin despite return of suprabasal MRLC phosphorylation to values that did not statisti-
cally differ from those induced by histamine alone. Ser$^{16}$-HSP20 phosphorylation remained elevated at 15 min. These data demonstrate increases in Ser$^{16}$-HSP20 phosphorylation during the sustained inhibitory phase of relaxation (2–15 min after addition of nitroglycerin). The sustained inhibitory phase was not associated with significant reductions in MRLC phosphorylation.

If Ser$^{16}$-HSP20 phosphorylation regulates the sustained inhibitory response, then Ser$^{16}$-HSP20 dephosphorylation should precede the recontraction observed when the relaxing agent is removed. Tissues were frozen for analysis when force had increased by 33 and 67% and when force reached steady state after washout of nitroglycerin in the continued presence of histamine. The data show that Ser$^{16}$-HSP20 phosphorylation decreased before force redevelopment (Fig. 3), as would be expected if the presence of Ser$^{16}$-HSP20 phosphorylation were responsible for the reduced force. Suprabasal MRLC phosphorylation values did not significantly change during force redevelopment after nitroglycerin washout. A significant increase in suprabasal MRLC phosphorylation would be expected if MRLC phosphorylation were regulating force redevelopment. Because this was not observed, we suggest that changes in suprabasal MRLC phosphorylation cannot be responsible for force redevelopment during nitroglycerin washout.

Comparison of nitroglycerin- and forskolin-induced relaxation. Ser$^{16}$-HSP20 phosphorylation was minimal at 0.006 ± 0.004 mol P/mol HSP20 in unstimulated

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**Fig. 3.** HSP20 phosphorylation (Phosphor) (shown in B) correlated with the sustained inhibitory phase of the nitroglycerin (NTG) response. Seven sets of swine carotid artery tissues were stimulated with 10 μM histamine alone for 30 min. Nitroglycerin (10 μM) was then added for 15 min and then washed out in the continued presence of histamine. A tissue from each set was frozen at 0 min for the unstimulated control, at 30 min with 10 μM histamine alone, and at 31, 32, and 45 min with 10 μM histamine plus nitroglycerin for 1, 2, and 15 min, respectively. After washout of nitroglycerin, the tissues were monitored for force redevelopment and were frozen when force redeveloped by 33% (actual time = 55.3 ± 1.6 min, 10.3 min after nitroglycerin washout), when force redeveloped by 67% (actual time = 60.7 ± 2.0 min, 15.7 min after nitroglycerin washout), and when force was stable (actual time = 74.8 ± 2.6 min, 24.8 min after nitroglycerin washout). After freezing, tissue homogenates were assayed for myosin regulatory light chains (MRLC; A) Ser$^{16}$-HSP20 (B) phosphorylation. For clarity, the first 30 min of histamine stimulation are abridged. The lines connecting control and 30 min histamine are typical for swine carotid artery, based on previous data (25). C: force was normalized to that elicited by 109 mM extracellular K$^+$ (K109) in the tissues. Results are means ± SE (n = 7 sets of tissues). Symbols are without error bars when SE was smaller than the symbol size.

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**Fig. 4.** Forskolin induces an inhibitory response that correlates with Ser$^{16}$-HSP20 phosphorylation but not MRLC phosphorylation. Five sets of swine carotid artery tissues were either 1) not stimulated (Cont), 2) stimulated with 10 μM histamine alone for 30 min (Hist), 3) stimulated with 10 μM histamine for 10 min and then relaxed by addition of 10 μM nitroglycerin for 20 min (H+NTG), or 4–7) stimulated with 10 μM histamine for 10 min and then relaxed by addition of 0.1, 0.3, 1.0 or 1.0 μM forskolin for 20 min. Contractile force was then measured, after which tissues were frozen for MRLC (A) and Ser$^{16}$-HSP20 (B) phosphorylation measurements. C: force was normalized to that elicited with 109 mM extracellular K$^+$ depolarization. Results are means ± SE (n = 5 set of tissues).
tissues (Fig. 4). Stimulation with a maximal concentration of histamine (10 μM) for 30 min contracted the tissues, increased suprabasal MRLC phosphorylation by 0.24 ± 0.04 mol P/mol MRLC, and did not significantly elevate Ser16-HSP20 phosphorylation. Addition of 10 μM nitroglycerin for the last 20 min of the histamine treatment relaxed the tissues and increased Ser16-HSP20 phosphorylation to 0.19 ± 0.02 mol P/mol HSP20, without significantly changing suprabasal MRLC phosphorylation.

Forskolin is a direct activator of adenyl cyclase. Addition of forskolin (0.1, 0.3, 1.0, and 10 μM) for the last 20 min of the histamine treatment induced dose-dependent sustained reductions in force (Fig. 4). Addition of forskolin was associated with suprabasal MRLC phosphorylation similar to that observed with histamine alone or histamine plus nitroglycerin. These data demonstrate that both forskolin and nitroglycerin induce relaxation without sustained MRLC dephosphorylation in these maximally histamine-stimulated tissues.

Forskolin-induced relaxation was associated with dose-dependent increases in Ser16-HSP20 phosphorylation (Fig. 4). The higher concentrations (1–10 μM) of forskolin increased Ser16-HSP20 phosphorylation to higher values and induced more complete relaxation that was observed with 10 μM nitroglycerin.

Figure 5 shows that Ser16-HSP20 phosphorylation induced by either forskolin or nitroglycerin correlated inversely with contractile force in 10 μM histamine-stimulated tissues.

**DISCUSSION**

Previously, smooth muscle relaxation was hypothesized to be a single process involving reversal of the contractile process. This interpretation was based on a paradigm derived from skeletal muscle in which relaxation is the reversal of excitation. This paradigm appears to explain smooth muscle relaxation resulting from withdrawal of excitatory stimuli. In these cases, reduction in [Ca2+]i-dependent MRLC phosphorylation precedes the reduction in force (9, 22, 29).

An important finding of this study is that “relaxation” of smooth muscle can involve two distinct phenomena with different regulatory mechanisms. Specifically, relaxation induced by agents that elevate cyclic nucleotides had two temporally distinguishable phases. The first is a rapid transient reduction in force characterized by reductions in [Ca2+]i-dependent MRLC phosphorylation (Fig. 3 and Ref. 18). The second element is manifested as a sustained inhibition of force that occurs despite return of MRLC phosphorylation to preinhibition levels. This is the “uncoupling of force from MRLC phosphorylation” noted in a previous study (1). It is this second element, sustained inhibition of force redevelopment, that may be mechanistically associated with Ser16-HSP20 phosphorylation.

We tested two predictions of this hypothesis. First, if Ser16 phosphorylation of HSP20 inhibits force during the sustained phase of cyclic nucleotide-induced relaxation, then 1) HSP20 should be phosphorylated during the sustained inhibition of force when MRLC phosphorylation is increasing and 2) Ser16-HSP20 dephosphorylation should precede a contraction induced by removal of the relaxing agent. Such a temporal correlation is one of the criteria described by Krebs and Beavo (15) in demonstrating the physiological relevance of a phosphorylation reaction. We found 1) that HSP20 became phosphorylated between 1 and 2 min after addition of nitroglycerin when MRLC phosphorylation began to increase and 2) that Ser16 dephosphorylation of HSP20 preceded force redevelopment induced by washing out nitroglycerin in the continued presence of histamine (Fig. 3). Suprabasal MRLC MRLC phosphorylation values did not significantly change during the force redevelopment, suggesting that changes in MRLC phosphorylation were not responsible for the change in force. These data are consistent with the hypothesis that Ser16-HSP20 phosphorylation is responsible for the nitroglycerin-induced sustained inhibition of force that occurs without MRLC dephosphorylation.

Importantly, Ser16-HSP20 phosphorylation did not correlate with the initial relaxation transient during which MRLC phosphorylation decreased significantly (Fig. 3). Previously, our laboratory (18) showed that nitroglycerin initially reduced [Ca2+]i, and MRLC phosphorylation in the swine carotid. These data suggest that nitroglycerin induced an initial relaxation transient that was mechanistically the reversal of excitation.

Second, if Ser16 phosphorylation of HSP20 regulates the sustained phase of force inhibition, there should be a unique relation between steady-state Ser16-HSP20 phosphorylation and reduction in force regardless of whether either cAMP or cGMP concentration is increased. We found that forskolin- and nitroglycerin-dependent increases in Ser16-HSP20 phosphorylation correlated with the sustained phase of force inhibition (Fig. 5). This is consistent with the hypothesis that...
Ser\textsuperscript{16}-HSP20 phosphorylation regulates the sustained phase of both cAMP- and cGMP-dependent relaxation without MRLC dephosphorylation. Importantly, Figs. 3 and 5 demonstrate a temporal and quantitative relationship between Ser\textsuperscript{16}-HSP20 phosphorylation and the sustained phase of force inhibition.

The results shown in Fig. 5 were plotted on a semi-logarithmic scale for clarity. Further work is required to define the quantitative relation between Ser\textsuperscript{16}-HSP20 phosphorylation and force inhibition. There was a suggestion that nitroglycerin relaxed the tissues slightly more for a given level of Ser\textsuperscript{16}-HSP20 phosphorylation than that observed with forskolin (as shown in Fig. 5). However, other preliminary experiments did not support this finding (O’Connor and Rembold, unpublished observations). Forskolin also relaxed tissues more than nitroglycerin. Higher concentrations of nitroglycerin did not induce complete relaxation (data not shown), probably because the enzymes that metabolize nitroglycerin to nitric oxide were saturated.

Some other stimuli, such as elevated extracellular Mg\textsuperscript{2+} concentration, induce smooth muscle relaxation without MRLC dephosphorylation (7). In the case of elevated Mg\textsuperscript{2+} concentration, the relaxation was not associated with Ser\textsuperscript{16}-HSP20 phosphorylation (26). These data suggest that Ser\textsuperscript{16}-HSP20 phosphorylation cannot fully explain the phenomenon of relaxation without MRLC dephosphorylation and that Ser\textsuperscript{16}-HSP20 phosphorylation is only one of perhaps multiple mechanisms that cause relaxation without MRLC dephosphorylation.

It is well established, both by our work (20) and others (16, 17), that cyclic nucleotides can induce relaxation by reducing [Ca\textsuperscript{2+}]i. The response depends on the relative concentrations of the excitatory stimulus and the inhibitory agent (18). It appears that reduction of [Ca\textsuperscript{2+}]i and MRLC phosphorylation suffice to the response of submaximal activated carotid media to forskolin or nitrovasodilators. Thus the precise response may be variable, depending on the tissue preparation, the type and concentration of the excitatory agonist, the type and concentration of inhibitory agent, and the time of measurement. We hypothesize that the mechanisms that maintain elevated [Ca\textsuperscript{2+}]i, are more susceptible to inhibition when smooth muscle is submaximally activated with submaximal increases in [Ca\textsuperscript{2+}]i. Maximal activation may more profoundly stimulate Ca\textsuperscript{2+} influx mechanisms so that increases in cAMP and/or cGMP concentration cannot substantially reduce [Ca\textsuperscript{2+}]i. It is possible that the “relaxation without MRLC dephosphorylation” mechanism exists so that force can be reduced even if cyclic nucleotides cannot reduce [Ca\textsuperscript{2+}]i and MRLC phosphorylation. On the basis of data present in this and previous studies (4, 24, 26), we propose that Ser\textsuperscript{16}-HSP20 phosphorylation may be such a mechanism.

Correlation of Ser\textsuperscript{16}-HSP20 phosphorylation and inhibition of force does not definitively establish cause and effect. Therefore, we must ask whether there is a plausible mechanism for HSP20 interfering with force generation. There are data suggesting that HSP20 binds to actin filaments (5, 24). We noted that a region of HSP20, contained in the peptide HSP20\textsubscript{10–121}, had a sequence homology similar to troponin I, the major thin filament regulatory protein in skeletal and cardiac muscle. HSP20\textsubscript{10–121} both reduced actin-activated myosin S1 ATPase activity and prevented contraction.

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Fig. 6. Working hypothesis for inhibition of smooth muscle tone by Ser\textsuperscript{16}-HSP20 phosphorylation. *Left:* currently accepted hypothesis for thick filament (i.e., myosin)-based regulation of smooth muscle contraction. An agonist, such as histamine, binds to a receptor and by various mechanisms (23) increases intracellular Ca\textsuperscript{2+} concentration. Ca\textsuperscript{2+} binds to calmodulin (CaM), activating myosin light chain kinase (MLCK), which phosphorylates myosin (M) on Ser\textsuperscript{19}. Phosphorylated myosin (Mp) has a higher actin-activated ATPase activity, which allows attachment to the actin (A) containing thin filaments. Attached phosphorylated cross bridges (Am) can either i) rapidly cycle (reforming Mp) and cause muscle shortening or 2) be dephosphorylated while attached, forming force-maintaining latch bridges (AM). Latch bridges can either i) slowly detach (forming M) or 2) be rephosphorylated to AMp and reenter the rapidly cycling pool. *Right:* our hypothesis for thin filament (i.e., actin)-based inhibition of smooth muscle force. Nitric oxide (NO) or nitrovasodilators activate soluble guanylyl cyclase producing cGMP. Hormones via receptors or forskolin activate adenyl cyclase producing cAMP. Both cAMP and cGMP appear to activate protein kinase G (PKG) in smooth muscle. PKG phosphorylates HSP20 on Ser\textsuperscript{16}. We hypothesize that Ser\textsuperscript{16}-phosphorylated HSP20 inactivates thin filaments. The result is fewer thin filaments available to cycle with phosphorylated myosin (Mp). Because fewer attached phosphorylated cross bridges (Am) are formed, we also hypothesize that fewer latch bridges (AM) would be produced. Therefore, force (the sum of Am and AM) is lower despite high MRLC phosphorylation. This model would predict that unloaded shortening velocity would not be affected by thin filament inactivation, since those phosphorylated cross bridges that attach to actin thin filaments would produce normal unloaded shortening. Some regulatory pathways are oversimplified here for reasons of clarity. Myosin dephosphorylation is accomplished by myosin light chain phosphatase (MLCP). There are multiple regulatory systems for MLCP (11) that alter the Ca\textsuperscript{2+} sensitivity of MRLC phosphorylation. The respective importance of PKG vs. protein kinase A in the phosphorylation of HSP20 has not yet been determined. The phosphorylation-specific response of HSP20 at the thin filament level is not characterized: Ser\textsuperscript{16}-HSP20 phosphorylation may cause a thin filament conformational change of previously bound HSP20 or may cause HSP20 association with (or even dissociation from) the thin filament. Finally, the identity of HSP20 phosphatase has not been identified.
We evaluated force in response to 10 μM histamine, a concentration that induces a maximal contraction. We thank Marcia Ripley for technical support and Mike Kurilla donated the swine carotid arteries. Our working hypothesis, shown in Fig. 6, states that HSP20, phosphorylated on Ser16, binds to and/or alters the conformation of thin filaments to inhibit attachment of phosphorylated cross bridges. If this were the case, increasing Ser16-HSP20 phosphorylation should reduce the amount of active force that can be generated at a given level of MRLC phosphorylation. This action could be cooperative such that a modest elevation in Ser16-HSP20 phosphorylation could maintain low tone. We evaluated force in response to 10 μM histamine, a concentration that induces a maximal contraction. We found that the amount of Ser16-HSP20 phosphorylation correlated with the maximal sustained contraction observed in the presence of 10 μM histamine (Fig. 5). These data support the hypothesis that Ser16-HSP20 phosphorylation is a mechanism responsible for relaxation without MRLC dephosphorylation. Figure 5 can also be interpreted to suggest that the level of Ser16-HSP20 phosphorylation determined the maximal level of force generated with contractile stimuli. This suggestion leads to the hypothesis that Ser16-HSP20 phosphorylation reduces force by inactivating thin filaments so as to remove parallel contractile units.

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