Heat shock protein 20-mediated force suppression in forskolin-relaxed swine carotid artery

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Meeks, Melissa K., Marcia L. Ripley, Zhicheng Jin, and Christopher M. Rembold. Heat shock protein 20-mediated force suppression in forskolin-relaxed swine carotid artery. Am J Physiol Cell Physiol 288: C633–C639, 2005. First published October 27, 2004; doi:10.1152/ajpcell.00269.2004.—Increases in cyclic nucleotide levels induce smooth muscle relaxation by deactivation [reductions in myosin regulatory light chain (MRLC) phosphorylation (e.g., by reduced \([\text{Ca}^{2+}]\)] or force suppression [reduction in force without reduction in MRLC phosphorylation]). Ser\(^{16}\)-heat shock protein 20 (HSP20) phosphorylation is the proposed mediator of force suppression. We evaluated three potential hypotheses whereby Ser\(^{16}\)-HSP20 phosphorylation could regulate smooth muscle force: \(1\) a threshold level of HSP20 phosphorylation could inactivate a thin filament as a whole, \(2\) phosphorylation of a single HSP20 could fully inactivate a small region of a thin filament, or \(3\) HSP20 phosphorylation could weakly inhibit myosin binding at either the thin- or thick-filament level. We tested these hypotheses by analyzing the dependence of force on Ser\(^{16}\)-HSP20 phosphorylation in swine carotid media. First, we determined that swine HSP20 has a second phosphorylation site at Ser\(^{157}\). Ser\(^{157}\)-HSP20 phosphorylation values were high and did not change during contractile activation or forskolin-induced relaxation. Forskolin significantly increased Ser\(^{16}\)-HSP20 phosphorylation. The relationship between Ser\(^{16}\)-HSP20 phosphorylation and force remained linear and was shifted downward in partially activated muscles relaxed with forskolin. Neither forskolin nor nitroglycerin induced actin depolymerization as detected using the F/G-actin ratio method in smooth muscle homogenates. These results suggest that force suppression does not occur in accordance with the first hypothesis (inactivation of a thin filament as a whole). Our data are more consistent with the second and third hypotheses that force suppression is mediated by full or partial inhibition of local myosin binding at the thin- or thick-filament level.

\(\text{cAMP; cGMP; nitric oxide; vascular smooth muscle}

Increases in cyclic nucleotide levels induce smooth muscle relaxation via two general mechanisms. One mechanism is deactivation, in which reductions in myoplasmic \([\text{Ca}^{2+}]\) concentration ([Ca\(^{2+}\)]) or increases in myosin phosphatase activity induce relaxation by reducing myosin regulatory light chain (MRLC) phosphorylation. The second mechanism is force suppression, in which there is a relaxation without a proportional reduction in [Ca\(^{2+}\)], or MRLC phosphorylation (13, 14, 17). Force suppression can be observed with increased cGMP concentration induced by nitric oxide (NO) (13), phosphodiesterase inhibitors (5), or increased cAMP concentration (20). Other treatments that induce force suppression include okadaic acid (22), some \([\text{Ca}^{2+}]/\text{depletion protocols (8), \text{Ca}^{2+}\] channel blockers (12), high extracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)], and other combinations of excitatory and inhibitory stimuli (2). Force suppression is not caused by phosphorylation of MRLC on amino acid residues other than Ser\(^{15}\) (13). NO-induced force suppression appears to involve increased cGMP concentration, given that a membrane-permeable cGMP analog also induces forced suppression (17).

Cyclic nucleotide-induced smooth muscle relaxation was found to correlate with phosphorylation of heat shock protein 20 (HSP20) on Ser\(^{16}\) (3, 4, 10, 26). Rembold and colleagues (17, 20) found that Ser\(^{16}\)-HSP20 phosphorylation correlated with force suppression rather than the deactivation form of relaxation. They noted that HSP20 has a sequence homology with troponin I: a peptide containing this homology bound to thin filaments, reduced actin-activated myosin S1 ATPase activity, and relaxed skinned smooth muscle (17). We hypothesized that binding of Ser\(^{16}\)-phosphorylated HSP20 to the thin filament turned “off” thin filaments so that phosphorylated myosin was unable to interact with the thin filament (i.e., a model similar to that for skeletal muscle troponin I). Such a model would explain reduced force despite MRLC phosphorylation.

If Ser\(^{16}\)-HSP20 phosphorylation regulates force suppression, then the dependence of relaxation on increases in Ser\(^{16}\)-HSP20 phosphorylation could suggest a mechanism(s) responsible for force suppression. There are three hypothetical mechanisms. The first hypothesis is that a threshold level of Ser\(^{16}\)-HSP20 phosphorylation could inactivate a thin filament as a whole, i.e., there could be cooperative inactivation of whole thin filaments by HSP20. This would be the expected result if Ser\(^{16}\)-HSP20 phosphorylation were to interfere with actin filament binding to cytoskeletal structures such as actinin, as proposed by Tessier et al. (23). It would also be the expected result if relaxation were to be associated with mechanically disrupted or depolymerized thin filaments. Contractile agonists have been reported to induce thin filament polymerization (16), suggesting the possibility that relaxation could be associated with reversal of thin filament polymerization. If this mechanism were operative, then increases in Ser\(^{16}\)-HSP20 phosphorylation should not alter force until a threshold is obtained. Above the threshold, further increases in Ser\(^{16}\)-HSP20 phosphorylation should significantly decrease force to near zero. A schema showing the predictions of this first hypothesis is shown in Fig. 1A.

A second hypothesis is that a small region of a thin filament could be fully inactivated by phosphorylation of a single HSP20 on Ser\(^{16}\), i.e., HSP20 regulates myosin binding locally. If so, then there should be a linear reduction in force with...
amounts of F- and G-actin. Finally, we also determined the relative amount of filamentous actin (F-actin) vs. globular-actin (G-actin) content in swine carotid arteries was determined with a commercial kit (BK037) from Cytoskeleton (Denver, CO). Lysis/F-actin stabilization buffer contained 50 mM KCl substituted stoichiometrically for NaCl. After relaxation in PSS, the rings were repeatedly stretched to ~20 g. The tissues were then released to ~5 g and recontracted with 109 mM [K\(^+\)], PSS. This protocol results in rings set within 5% of their optimal length (18). The second 109 mM [K\(^+\)]\(_{0}\) PSS contraction was used for normalizing further forces. After relaxation in PSS, the length of each ring was measured. Rings were then (1) untreated (control); (2) activated with 1 or 10 \(\mu\)M histamine in PSS for 60 min; (3) activated with histamine in PSS for 30 min and then relaxed by the addition of 0.1, 0.3, 1, 3, or 10 \(\mu\)M forskolin for 60 min. The longer times were required with [K\(^-\)], depolarization to attain stable steady-state force values. Rings were then frozen in an acetonitrile-dry ice slurry. After air drying, the tissues were weighed and homogenized in a buffer containing 1% SDS, 10% glycerol, 20 mM dithiothreitol, and 0.05% bromophenol blue (20 mg wet wt/ml buffer).

Measurement of HSP20 and MRLC phosphorylation. Rabbit anti-HSP20 antibody was made commercially via repeated injection of gel-purified recombinant HSP20 (sequence confirmed by mass spectrometry). After confirmation of an antigenic response, serum was collected and frozen for future use. Rabbit anti-MRLC antibodies were a gift from Subah Packer. 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 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, where pl is the isoelectric point), 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 (ECL) (19). The dilutions ensured that the ECL detection system was in the linear range (19). Immunoblots were imaged with a digital camera and quantituated with UnScanIt software.

Cyclic nucleotide assays. Swine carotid rings were frozen as described in Tissues, homogenized in 0.1 M HCl, and analyzed for cAMP and cGMP by radioimmunoassay as described previously (15). HSP20 phosphorylation sites. Unstimulated swine carotid rings were frozen as described in Tissues, homogenized, and centrifuged at 14,000 g for 10 min, and the supernatant proteins were separated first by isoelectric focusing (pl range 5–7) followed by 12% polyacrylamide gel electrophoresis (1, 7). Coomassie blue-stained bands containing HSP20 were excised from heavily loaded two-dimensional gels, minced, destained, alkylated, and trypsinized as described previously (9). Peptides were then run on a POROS 10 RC reversed-phase microcapillary HPLC, and its output was directed into a Finnigan-MAT TSQ7000 electrospray tandem mass spectroscope (9). Collisionally activated dissociation spectra were interpreted, and the proposed peptide fragment sequences were then compared with published protein sequences.

F/G-actin content. The relative amount of filamentous actin (F-actin) vs. globular-actin (G-actin) content in swine carotid arteries was determined with a commercial kit (BK037) from Cytoskeleton (Denver, CO). Lysis/F-actin stabilization buffer contained 50 mM pipera-
Identification of HSP20 phosphorylation sites. Figure 2 shows a two-dimensional gel from a swine carotid tissue that was not treated with agents that increase cAMP or cGMP concentration. The spot labeled S157 was excised and sequenced using mass spectroscopy; this spot was identified as HSP20 that was monophosphorylated on Ser157. Table 1 shows the predicted human, rat, and swine protein sequences based on published DNA sequences compared with the mass spectroscopy sequence labeled “protein.” A mass spectroscopy sequence was obtained on all but 24 residues of HSP20 (the missing residues are shown in lowercase). The only difference between the sequenced protein and the predicted sequence from DNA sequencing was at residue 64: a valine in the protein sequence vs. a threonine in the DNA sequence. Swine HSP20 is similar to rat HSP20, which was previously shown to have Ser157 phosphorylation site.

Table 1. HSP20 sequences predicted from DNA and from mass spectroscopy sequencing including phosphorylation sites

<table>
<thead>
<tr>
<th>Species</th>
<th>HSP20 Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>MEI PV PQS WLR ASA LPLG SAP GLF DRQ FGE GL L E A L C TT L A P Y L</td>
</tr>
<tr>
<td>Bovine</td>
<td>MEI PV PQS WLR ASA LPLG SAP GLF DRQ FGE GL L E A L C TA A P Y L</td>
</tr>
<tr>
<td>Rat</td>
<td>MEI PV PQS WLR ASA LPLG FST PDRQ FGE GL L E A L C TA A P Y L</td>
</tr>
<tr>
<td>Mouse</td>
<td>MEI PV PQS WLR ASA LPLG SAP GLF DRQ FGE GL L E A L C TA A P Y L</td>
</tr>
<tr>
<td>Swine</td>
<td>MEI PV PQS WLR ASA LPLG SAP GLF DRQ FGE GL L E A L C TA A P Y L</td>
</tr>
<tr>
<td>MS</td>
<td>Ac- MEI PV PQS WLR ASA LPLG SAP GLF DRQ FGE GL L E A L C TA A P Y L</td>
</tr>
</tbody>
</table>

Ser16 phosphorylation site (PKA/PKG site)

<table>
<thead>
<tr>
<th>Species</th>
<th>HSP20 Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>RAP SVA LP VQA QTP DGP HFS VLD V HSPE EI AVK VG H VHE V HAE H R P E D H</td>
</tr>
<tr>
<td>Bovine</td>
<td>RAP SVA LP VQA QTP DGP HFS VLD V HSPE EI AVK VG H VHE V HAE H R P E D H</td>
</tr>
<tr>
<td>Rat</td>
<td>RAP SVA LP VQA QTP DGP HFS VLD V HSPE EI AVK VG H VHE V HAE H R P E D H</td>
</tr>
<tr>
<td>Mouse</td>
<td>RAP SVA LP VQA QTP DGP HFS VLD V HSPE EI AVK VG H VHE V HAE H R P E D H</td>
</tr>
<tr>
<td>Swine</td>
<td>RAPS V A LP T Q D P HFS VLD V HSPE EI AVK VG H VHE V HAE H R P E D H</td>
</tr>
<tr>
<td>MS</td>
<td>RAPS V A LP T Q D P HFS VLD V HSPE EI AVK VG H VHE V HAE H R P E D H</td>
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Ser157 phosphorylation site

Human sequence is predicted from NCBI database 014558; bovine sequence is predicted from www.tigr.org BM968020 and BM89715; rat sequence is predicted from NCBI database A53814; mouse sequence is predicted from NCBI database XP145511; and swine sequence is predicted from www.tigr.org TC95752. MS is mass spectroscopy protein sequence of swine HSP20. Bold letters represent homology; lowercase letters are missing mass spectroscopy sequence; # indicate the two phosphorylation sites; underlined sequences are the PKA/PKG site at Ser16 and the Tn1 homology site, respectively.

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three extra residues (compared with human) at locations 155–157, including a serine at 157 (25). These three extra residues, including Ser157, were also present in mouse and bovine HSP20. Figure 1 has other spots that were previously identified using mass spectroscopy (17). Of interest is the circle in Fig. 2 labeled S16 and S157 showing the location of the spot previously identified as Ser16-phosphorylated HSP20. This spot likely represents HSP20 diphosphorylation at both Ser16 and Ser157 (3, 20); however, prior sequencing did not include Ser157.

Biochemical events during forskolin-induced relaxation. We measured the steady-state relationships among HSP20 phosphorylation, MRLC phosphorylation, and force in maximally and submaximally stimulated swine carotid artery relaxed using various concentrations of forskolin, a direct activator of adenylyl cyclase. Figure 3 shows all the biochemical measures and force data plotted against forskolin dose, and Fig. 4 shows the dependence of force on Ser19-MRLC phosphorylation.

Figure 4A shows the relationship between Ser19-MRLC phosphorylation and force induced by histamine or high [K+]o. Unstimulated (control) tissues had low force and Ser19-MRLC phosphorylation values of 0.14 ± 0.02 mol P/mol MRLC. Activation with histamine (Fig. 4A, filled diamonds) or high [K+]o, depolarized (open diamonds) increased both force and Ser19-MRLC phosphorylation such that a sigmoidal dependence of force on Ser19-MRLC phosphorylation was observed. A Hill curve fit revealed half-maximal force when Ser19-MRLC phosphorylation was 0.20 mol ± 0.01 P/mol MRLC. This relationship allows us to define forskolin-induced force suppression.

Increasing forskolin concentration caused dose-dependent reductions in force without significant changes in Ser19-MRLC phosphorylation (Fig. 3, C and D). Force suppression is demonstrated as a MRLC phosphorylation-force value significantly below (or to the right of) the sigmoidal dependence of force on Ser19-MRLC phosphorylation as shown in Fig. 4A. Addition of forskolin to histamine-stimulated tissues reduced force significantly below the regression line consistent with force suppression (Fig. 4B, filled circles). Addition of forskolin to high-[K+]o-depolarized tissues also reduced force so that the mean force values fell primarily below the line; however, most of the error bars included the regression line, so force suppression was not clearly demonstrable (Fig. 4B, open circles). Because high [K+]o alone induced MRLC phosphorylation values on the steep portion of the force-MRLC phosphorylation relationship, it was difficult to demonstrate force suppression as a downward shift in the relationship.

Ser157-HSP20 phosphorylation values were high in resting tissues and did not change with histamine stimulation or high-[K+]o depolarization (Fig. 3B). Addition of forskolin appeared to slightly decrease Ser157-HSP20 phosphorylation, although values remained quite high.

Ser16-HSP20 phosphorylation values were low in resting tissues and did not change with histamine stimulation or high-[K+]o depolarization (Fig. 3A). Increasing forskolin concentration increased Ser16-HSP20 phosphorylation and relaxed swine carotid artery in a dose-dependent manner. The concentration of histamine that activated the tissues did not affect forskolin-induced increases in Ser16-HSP20 phosphorylation. Similarly, the level of high-[K+]o depolarization did not affect forskolin-induced increases in Ser16-HSP20 phosphorylation.

However, compared with high-[K+]o-depolarized tissues, the histamine-stimulated tissues had higher Ser16-HSP20 phosphorylation values when relaxed with 0.3 and 1 μM forskolin (dotted line in Fig. 3A, right).

The dependence of force on Ser16-HSP20 phosphorylation is detailed in Fig. 5 and Table 2. When tissues were activated by 1 or 10 μM histamine or 25 or 30 mM [K+]o, addition of forskolin produced a similar slope and x (HSP20 phosphorylation)-intercept in the dependence of force on Ser16-HSP20 (Table 2). When tissues were activated with 40 mM [K+]o, the dependence of force on Ser16-HSP20 phosphorylation had a significantly greater x (HSP20 phosphorylation)-intercept than...
during the other four stimulation protocols (slope appeared lower but did not reach statistical significance, $P = 0.051$).

**F-actin changes during cyclic nucleotide-induced relaxation.** Stimulation of swine carotid artery with 10 μM histamine significantly increased the relative amount of F-actin, decreased G-actin, increased Ser19-MRLC phosphorylation, increased stress, and did not change cAMP or cGMP concentration (Fig. 6). This increase in F-actin confirms prior studies in dog trachealis (16). Addition of 100 μM nitroglycerin or 1 μM forskolin to 10 μM histamine-stimulated tissues induced a significant relaxation that was not associated with a significant change in the relative amounts of F- or G-actin compared with histamine alone (Fig. 6, A and B). Addition of nitroglycerin to histamine-stimulated tissues significantly increased cGMP concentration without changing cAMP concentration (Fig 6C). Addition of forskolin to histamine-stimulated tissues significantly increased cAMP concentration without changing cGMP concentration. Addition of either forskolin or nitroglycerin to histamine-stimulated tissues was associated with force suppression (Fig. 6D, inset).

![Fig. 4. Dependence of contractile stress on Ser16-HSP20 phosphorylation in swine carotid artery. A: dependence of stress on Ser16-HSP20 phosphorylation in tissues stimulated with 0.3, 1, 3, or 10 μM histamine (filled diamonds) or depolarized with 40, 30, or 25 mM [K+]o (open diamonds; data are from Fig. 3). These data were fit with a Hill plot, which is shown as a solid line (regression equation stress = 1.12 × M₁⁰²⁰/[0.0281 + M₁⁰²⁰]), where M₁ is MRLC phosphorylation; $r^2 = 0.88$). B: dependence of stress on Ser16-MRLC phosphorylation in tissues stimulated with histamine and relaxed with forskolin (filled circles) or depolarized with [K+]o and relaxed with forskolin (open circles; data are from Fig. 3). The solid line represents the regression from data for histamine and [K+]o, alone from Fig. 3, A. Data are presented as means ± SE with $n = 4–7$. Some error bars are obscured by symbols.

**DISCUSSION**

Our goal was to evaluate the mechanism whereby Ser16-HSP20 phosphorylation regulates smooth muscle force by analyzing the dependence of force on Ser16-HSP20 phosphorylation with partial activation of swine carotid media. This analysis was predicated on the general hypothesis that Ser16-HSP20 phosphorylation is the mediator of force suppression.

Our first hypothesis, that HSP20 mediates full inactivation of thin filaments, predicts a sigmoidal dependence of force on Ser16-HSP20 phosphorylation (see schema in Fig. 1). This was not observed (Fig. 5), and therefore, the first hypothesis (full inactivation of thin filaments) is not supported. These data therefore suggest that Ser16-HSP20 phosphorylation-induced relaxation is not likely to act via disruption of the linkage of thin filaments to cytoskeletal structures (23).

Prior studies suggested that contractile activation is associated with thin filament polymerization (16). We found that

<table>
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<tr>
<th>Stimuli</th>
<th>Slope</th>
<th>Ser16-HSP20 Phosphorylation (x) Intercept</th>
<th>$r^2$</th>
<th>$n$</th>
</tr>
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<tbody>
<tr>
<td>[K+]o (40 mM)</td>
<td>$-0.56±0.06$</td>
<td>$1.73±0.12^*$</td>
<td>0.82</td>
<td>5</td>
</tr>
<tr>
<td>[K+]o (30 mM)</td>
<td>$-0.91±0.14$</td>
<td>$1.01±0.16$</td>
<td>0.94</td>
<td>4</td>
</tr>
<tr>
<td>[K+]o (25 mM)</td>
<td>$-0.91±0.20$</td>
<td>$0.79±0.14$</td>
<td>0.87</td>
<td>4</td>
</tr>
<tr>
<td>Histamine (10 μM)</td>
<td>$-1.23±0.17$</td>
<td>$1.04±0.08$</td>
<td>0.90</td>
<td>5</td>
</tr>
<tr>
<td>Histamine (1 μM)</td>
<td>$-0.98±0.17$</td>
<td>$0.82±0.02$</td>
<td>0.90</td>
<td>4</td>
</tr>
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</table>

Data from Fig. 5 were analyzed using regression analysis. Data from each artery (e.g., histamine at 10 μM alone and with forskolin at 0.3, 1, 3, and 10 μM) were analyzed separately with regression analysis. The regression results were then averaged for each treatment group and are presented as means ± SE ($r^2$ is mean only). Slope is in units of force (%109 mM [K+]o)/Ser16-HSP20 phosphorylation (mol P/mol HSP20). The x-intercept is in units of Ser16-HSP20 phosphorylation (mol P/mol HSP20). Data were compared using one-way ANOVA. * $P < 0.001$ compared with the other 4 treatments.
histamine stimulation of swine carotid artery increases the relative amount of F-actin compared with G-actin and is in agreement with this prior report (Fig. 6). Of interest, we employed a different method in a different tissue, suggesting that the increase in F-actin with activation is robust. We performed this analysis to test the hypothesis that force suppression was a reversal of this process, i.e., it was associated with F-actin depolymerization. This was not observed (Fig. 6). Relaxation induced by nitroglycerin or forskolin induced force suppression (Fig. 6D, inset) but did not alter the relative amount of F- or G-actin compared with histamine stimulation alone. Data for both the dependence of force on Ser$^{16}$-HSP20 phosphorylation (Fig. 5) and the F/G-actin polymerization (Fig. 6) suggest that force suppression is not caused by a large depolymerization of thin filaments. It should be noted that the F/G-actin ratio will not detect changes in the structure of F-actin if changes do not alter the total amount of F-actin.

The second hypothesis, that HSP20 mediates regional activation of thin filaments, predicts that there should be a common x-intercept in the dependence of force on Ser$^{16}$-HSP20 phosphorylation. We found similar x (HSP20 phosphorylation)-intercepts in tissues activated with 1 or 10 μM histamine or 25 or 30 mM [K$^+$]o, and then relaxed with forskolin. Tissues activated with 40 mM [K$^+$]o had a significantly higher x (HSP20 phosphorylation)-intercept than tissues stimulated with the other four stimuli. It is possible that the higher [Ca$^{2+}$]o, induced by 40 mM [K$^+$]o, could be responsible for the increase in the x-intercept. This is the subject of further study.

The third hypothesis, that HSP20 mediates weak inhibition of myosin binding at either the thin- or thick-filament level, predicts that there should be a common slope of the dependence of force on Ser$^{16}$-HSP20 phosphorylation. We did not find significantly different slopes with the five stimuli that were relaxed with forskolin, although it should be noted that the P value for the ANOVA was 0.051, suggesting the possibility of a type II error.

Our data did not clearly delineate between the second and third hypotheses. These data suggest that Ser$^{16}$-HSP20 phosphorylation reduces force by either regional thin filament inhibition or weak inhibition of myosin binding at the thin- or thick-filament level. Our data do not allow us to determine whether this effect occurs at the level of cross-bridge attachment or maintenance of bound cross bridges. Further research is required to determine how such a mechanism could function.

We determined that the second phosphorylation site on swine carotid was Ser$^{157}$. This is the site identified in rat platelets by Wang et al. (25) and is likely to be the same site of phosphorylation described by Beall et al. (3) in the COOH terminus of HSP20. In platelets, Ser$^{157}$-HSP20 phosphorylation was mediated by insulin (25). In the swine carotid artery, Ser$^{157}$-HSP20 phosphorylation was high and remained high with activation and with forskolin-induced relaxation. There was no clear relationship between Ser$^{157}$-HSP20 phosphorylation and force (Fig. 3). Interestingly, Ser$^{157}$ is not present in human HSP20 (11).

When tissues were activated with histamine, forskolin-induced relaxation was clearly associated with force suppression (Fig. 4). However, when tissues were depolarized with high [K$^+$]o, forskolin-induced force suppression was more difficult to measure. This resulted from the steepness of the Ser$^{19}$ MRLC phosphorylation-force relationship. Therefore, minor inaccuracies in measurement of Ser$^{19}$ MRLC phosphorylation make it difficult to analyze the degree of force suppression in [K$^+$]o-depolarized tissues. This result does not imply that force suppression does not occur when tissues are depolarized with high [K$^+$]o; it suggests that it is difficult to measure. The steepness of the Ser$^{19}$-MRLC phosphorylation-force relation-
ship suggests the presence of cooperativity in the regulation of force by Ser\textsuperscript{19}-MRLC phosphorylation: this is the subject of another investigation by investigators in our laboratory (21).

Our results confirm that forskolin induces force suppression in the histamine-stimulated swine carotid. If Ser\textsuperscript{16}-HSP20 phosphorylation is found to be the mediator of force suppression, we propose that the mechanism for this force suppression is regional thin filament inhibition or weak inhibition of myosin binding at the thin or thick filament. We also found that the second phosphorylation site of swine HSP20 is Ser\textsuperscript{157}.

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GRANTS

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