Smooth muscle myosin is activated by regulatory light chain (RLC) phosphorylation. In the unphosphorylated state the activity of both heads is suppressed due to an asymmetric, intramolecular interaction between the heads. The properties of myosin with only one of its two RLCs phosphorylated, a state likely to be present both during the activation and the relaxation phase of smooth muscle, is less certain despite much investigation. Here we further characterize the mechanical properties of an expressed heavy meromyosin (HMM) construct with only one of its RLCs phosphorylated (HMM-1P). This construct was previously shown to have more than 50% of the ATPase activity of fully phosphorylated myosin (HMM-2P) and to move actin at the same speed in a motility assay as HMM-2P (Rovner, A. S., Fagnant, P. M., and Trybus, K. M. (2006) Biochemistry 45, 5280–5289). Here we show that the unitary step size and attachment time to actin of HMM-1P is indistinguishable from that of HMM-2P. Force-velocity measurements on small ensembles show that HMM-1P can generate approximately half the force of HMM-2P, which may relate to the observed duty ratio of HMM-1P being approximately half that of HMM-2P. Therefore, single-phosphorylated smooth muscle HMM molecules are active species, and the head associated with the unphosphorylated RLC is mechanically competent, allowing it to make a substantial contribution to both motion and force generation during smooth muscle contraction.

Myosin motors are involved in a diverse array of actin-based cellular functions including muscle contraction, cargo transport, and cytokinesis. To accomplish any of these processes successfully, there needs to be strict control of when the motor is activated and when it is turned “off.” Smooth muscle myosin, which powers smooth muscle contraction in both vascular and visceral tissues, is no exception, and the mechanism by which it is activated and when it is turned “off.” Smooth muscle myosin, which is regulated has been studied for many years (for review, see Ref. 2). Smooth muscle myosin is activated when the calcium-calmodulin-myosin light chain kinase complex phosphorylates Ser-19 of the regulatory light chain (RLC) bound to the neck of the myosin head. In the unphosphorylated state, smooth muscle myosin is unable to move actin, and the actomyosin ATPase activity is rate-limited by phosphate release so that the motor can only weakly interact with actin in the M-ADP-P state (3).

Early studies characterized the inhibited state of myosin at physiologic ionic strength as a species that sedimented at 10 S in the ultracentrifuge, indicating that the rod must adopt a compact conformation (4, 5). Consistent with the hydrodynamic studies, metal-shadowed images showed a structure with the rod bent into nearly equal thirds and heads bent back toward the rod (6). Higher resolution cryo-electron microscopic images of two-dimensional arrays of unphosphorylated HMM revealed an asymmetric intramolecular interaction between the heads called the “blocked” and “free” heads that proposed a molecular basis for inhibition (7). The actin binding domain of the blocked head interacts with the converter domain of the free head, so that the blocked head cannot bind actin and be actin-activated. The free head is prevented from progressing through its ATPase cycle because rotation of the converter domain cannot occur due to the binding of the blocked head, and thus, the free head is locked in a weak binding state (7). These asymmetric head interactions were also observed by single particle analysis of negatively stained images of smooth muscle myosin (8). This motif appears to be a general mechanism widely used by class II myosins to maintain a relaxed or inhibited state, as it was also observed in native striated muscle myosin thick filaments from tarantula, which are regulated by phosphorylation (9), as well as in striated myosins from both vertebrates and invertebrates (10).

RLC phosphorylation abolishes these interactions, allowing both heads to freely interact with actin (7, 11). Although these two endpoints are well characterized, much less is agreed upon with regard to smooth muscle myosin that has only one of its two RLCs phosphorylated. RLC phosphorylation by myosin light chain kinase is random (12–14), so myosin with only one phosphorylated RLC is a predominant species during muscle activation and perhaps during relaxation. The hydrolytic and mechanical activity of this state has been investigated for decades. In the early studies, the activity of single-phosphorylated myosin was inferred from ensemble measurements in which it existed in a mixture with both unphosphorylated and double-phosphorylated myosin. Some of these studies suggested that it has less than half the actin-activated ATPase activity of the double-phosphorylated state (15, 16), whereas others suggested that both the hydrolytic and actin filament motility was approximately half (17, 18). The former studies imply that the activation of one head does not activate the...
whole molecule, whereas the latter was consistent with each head acting independently of its partner.

Recently, the approach to this problem has been improved by employing various methods that allow isolation of a single-phosphorylated species (1, 19, 20). Single-phosphorylated heavy meromyosin (HMM) had much less than half the hydrolytic and mechanical activity of double-phosphorylated HMM when prepared using light chain exchange or stripping protocols (19, 20). Using differential tagging of constructs expressed in Sf9 cells followed by sequential affinity columns, the single-phosphorylated HMM (HMM-1P) had more than half the ATPase activity and actin filament speeds in the in vitro motility assay that were similar to double-phosphorylated HMM (1).

Here, we further characterize the mechanical properties of the expressed HMM-1P construct. An optical trap assay was used to show that the unitary step size and attachment time of an expressed single HMM-1P molecule was indistinguishable from that of double-phosphorylated HMM (HMM-2P) (1), suggesting that at least one of the heads of HMM-1P is equivalent to a head of HMM-2P. The optical trap was further used to characterize the force-velocity relationship for a small ensemble of HMM-1P molecules (21). These data showed that HMM-1P can generate approximately half the force of HMM-2P, which may relate to the observed duty ratio of HMM-1P being approximately half that of HMM-2P. The results are discussed in terms of two mechanisms that cannot be distinguished from one another based on the current data. The ability of HMM-1P to generate motion and force implies that it likely contributes to smooth muscle contraction both during activation at low phosphorylation levels as well as in maintaining tension when phosphorylation levels start to decline.

EXPERIMENTAL PROCEDURES

HMM Constructs—The heavy chain of the double-headed heavy meromyosin construct used here was truncated after Arg-1175 followed by a Gly-Ser linker and then an 88-amino acid segment from the Escherichia coli biotin carboxyl carrier protein. This length of tail ensures formation of a stable α-helical coiled-coil. A FLAG tag followed the biotin tag on the heavy chain and was used to facilitate purification of the HMM-2P on a FLAG affinity column. The only difference between the proteins used here and those extensively characterized in Rovner et al. (1) is the presence of a biotin tag. The biotin tag was retained for specific attachment of all proteins to a neutravidin-coated coverslip. The biotin carboxyl carrier protein is biotinated in the Sf9 cells, as described previously (22). For all constructs containing the biotin tag, the media was supplemented by 0.2 mg biotin/ml media after infection with recombinant baculovirus.

The construct that contained a single-phosphorylated head was prepared as described previously (1), with the exception that the HMM heavy chain contained the biotin tag described above but with no FLAG tag after it. His-tagged wild-type and FLAG-tagged T18A/S19A regulatory light chains were co-infected with this heavy chain construct followed by sequential affinity columns to isolate a homogeneous population of heavy meromyosin containing one wild-type light chain and one T18A/S19A light chain. The tags on the light chains were removed by thrombin cleavage, and the preparation was phosphorylated with myosin light chain kinase before functional analysis as described previously (1). SDS and charge gel electrophoresis were used to confirm that equal amounts of the two light chains were present and that the final preparation was 50% phosphorylated as described in detail in Rovner et al. (1). The use of the T18A/S19A mutant light chain as a mimic of the unphosphorylated state is supported by the fact that a preparation of HMM containing two non-phosphorylatable mutant light chains was unable to move actin in a motility assay (see Fig. 7A of Rovner et al. (1)).

HMM Surface Attachment and Density—Each HMM construct was attached to a neutravidin (NAV)-coated flow cell surface through its C-terminal biotin tag. This attachment strategy allowed us to effectively control the HMM surface density by varying the NAV surface density (see the supplemental material for details).

To estimate the extent to which HMM binds to the NAV surface, we measured the amount of fluorescent actin “shards” (i.e. very short actin filaments) bound to rigor HMM (i.e. in the absence of ATP) as an indicator of the HMM density on the NAV surface. We performed this assay at a constant HMM-1P concentration (100 μg/ml), whereas the surface was prepared with varying NAV concentrations between 0 and 200 μg/ml (Fig. 2, a and b). We assessed the actin binding in this manner using only the HMM-1P, as rigor binding is independent of the HMM phosphorylation state (23) and, thus, should be the same regardless of the HMM construct used. Flow cells (15–20 μl volume), prepared as described previously (24), were incubated for 2 min with NAV (Invitrogen) (0–200 μg/ml) diluted in actin buffer (AB) consisting of 25 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM dithiothreitol, 0.25 μg/ml glucose oxidase, and 45 μg/ml catalase at pH 7.4. Subsequently, the surface was washed 5 times with AB to remove unbound NAV and blocked with 0.5 mg/ml bovine serum albumin in AB for 6 min, after which 100 μg/ml HMM-1P was added and incubated for 2 min. The flow cells were then washed two times with AB. Shredded actin filaments (0.4 μg/ml) in AB with actin purified from chicken pectoralis and labeled with TRITC-phalloidin (24) were vortexed and sonicated to create tiny filament shards just before being added to the flow cell. The actin was then incubated for 5 min in the absence of ATP followed by 5 washes with AB. Actin shards bound tightly to HMM on the flow cell surface and were imaged (Fig. 2a) through an objective-type total internal reflectance fluorescence microscope and recorded (25).

Four still frames, each a 10-frame average at a different location, were recorded from each flow cell with the camera settings (i.e. gain and 67-ms integration time) kept constant for all conditions. The overall actin filament fluorescence intensity was measured as an index of HMM binding to the surface. Based on these data (see Fig. 2b), by varying the NAV concentrations below 50 μg/ml and keeping the HMM concentration constant at 100 μg/ml, we could control the HMM surface density in the linear regime below surface saturation.

Single HMM Molecule Optical Trapping—We measured the step size (d) and attachment time (tatt) for single HMM molecules using an optical trap (26). To attach beads to both ends of
Single-phosphorylated Smooth Muscle HMM

an actin filament, which serve as “handles” in the optical trap, we first coated 1-µm-diameter silica beads (Bangs Laboratories, Inc.) with N-ethylmaleimide (NEM)-modified myosin by overnight incubation at room temperature. Excess NEM-modified myosin was removed from the silica beads and the solution by washing with AB, sonication, and centrifugation at 10,000 rpm for 1 min. The beads were resuspended in AB, and the process was repeated seven times.

To eliminate inactive heads before each experiment, HMM was centrifuged at 95,000 rpm in an equimolar actin solution with 1 mM MgATP. All HMM concentrations reported are the concentration before centrifugation. Approximately 20–30% of the HMM was lost in this treatment.

Flow cells for the optical trap experiments were prepared as described previously (26, 27), except that 3-µm-diameter beads were used as HMM pedestals. To operate in the single HMM molecule regime, we incubated the flow cell with limiting concentrations of NAV (0.05–0.2 µg/ml) in AB for 2 min followed by 2 AB washes, 0.5 mg/ml bovine serum albumin in AB for 6 min, 100 µg/ml HMM construct for 2 min, 2 AB washes, and finally, NEM-modified myosin-coated beads, TRITC-actin filaments, and 10 µM ATP in AB. These experiments were performed at room temperature, ~20 °C.

The flow cell was then placed on the microscope stage, and an NEM bead was captured in each of the two traps. We then affinity either end of a TRITC-actin filament to the NEM beads, pre-tensioned the filament, and calibrated the system (26). The bead-actin-bead assembly was then lowered onto a HMM-coated bead pedestal to record single HMM molecule binding events. The displacement traces were then analyzed using the mean-variance method (28, 29).

Duty Ratio—To estimate the HMM duty ratio, i.e. the proportion of the cross-bridge cycle that the HMM is strongly bound to actin, the actin filament speed was measured for each actin filament as was its length (30, 31) at low HMM surface densities (15–40 µg/ml NAV, 100 µg/ml HMM) at room temperature ~20 °C. For each actin filament, we measured its instantaneous velocity between frames and then calculated an average velocity over its entire motion path (31). Based on the relationships between actin filament speed versus actin filament length at different HMM surface densities, we could estimate the duty ratio (α) for the HMM-1P and -2P constructs using the equation (30, 31),

\[
V = aV_{\text{max}} (1 - (1 - \alpha)^N) \quad \text{(Eq. 1)}
\]

where \(N\) is the number of HMM heads capable of interacting with the actin filament (note that \(N = L/L_0\), where \(L\) is the actin filament length, and \(L_0\) is the average spacing between HMM heads on the motility surface). \(V_{\text{max}}\) is the maximum average speed when at least one HMM head translates the actin filament at any point in time, and \(a\) is an empirical “efficiency factor.” One interpretation of \(a\) is that it accounts for energy lost to buckling the actin filament when the spacing between HMM molecules on the surface is large (30). An alternate interpretation is that it accounts for the length-dependent viscous drag of a filament moving through a fluid. Thus, at high HMM concentrations more HMM molecules interact with an actin filament of a given length, so this drag force is shared between them and has a lesser effect.

Force-Velocity—Force-velocity data were collected for small ensembles of HMM as previously described (21). To perform these experiments, the single molecule optical trap assay was modified to support large loads. In particular, the 1-µm silica beads used to manipulate the actin filaments were replaced by 1.4-µm diameter polystyrene beads, which resulted in a larger linear range on the quadrant detector and a stiffer trap (i.e. greater trapping forces >10 pN). In the single molecule assay described above, actin filaments are attached to the beads only through an NEM-myosin linkage. However, to withstand the larger forces in this force-velocity assay and to prevent the actin filament from being ripped off the beads, actin filaments were attached to the beads via the usual NEM-myosin linkage with an additional linkage through NAV-biotin conjugation. Therefore, beads were incubated in a mixture of 10 mg/ml NAV and 1.4 µg/ml NEM-myosin overnight as described above. We then used actin filaments incubated with TRITC-phalloidin (Sigma-Aldrich) and biotin-labeled phalloidin (Invitrogen) at a 2:1:1 actin:TRITC-phalloidin:biotin-phalloidin molar ratio.

Flow cells were constructed as for the single molecule assay but with higher HMM surface densities required for these ensemble measurements. Thus, flow cells were incubated for 2 min with 40 µg/ml NAV in AB, washed, and then blocked with 0.5 mg/ml bovine serum albumin in HB6 min followed by a 2-min 100 µg/ml HMM incubation. We then additionally blocked the NAV surface with 1 mg/ml biotinated-bovine serum albumin (Pierce) in AB for 6 min to prevent any unbound NAV from interacting with the trapped biotinated TRITC-actin filament. The experiments were conducted at 100 µM ATP and at room temperature ~20 °C.

In contrast to the HMM-2P, 40 µg/ml NAV did not provide enough HMM-1P on the surface to generate and sustain the required forces to perform this assay. Even using 80 µg/ml NAV to saturate the surface with HMM-1P (see Fig. 2b) was still inadequate. To circumvent this problem, the amount of HMM-1P available to interact with the actin filament was increased 1.6 times by using larger, 5.0-µm-diameter bead pedestals on the flow cell surface (see supplemental Fig. S2). Under these conditions, HMM-1P force-velocity measurements were feasible.

To obtain force-velocity relationships, constant loads of 1–10 pN were applied to the actin filament against which the HMM ensemble was forced to operate (21). The protocol involved a staircase of loads (1–6, 8, and 10 pN) with individual loads applied for 200–1000 ms and with the larger loads applied

| Construct | Step size (nm) | Attachment time (ms) |
|-----------|---------------|----------------------|
| HMM-2P    | 11.4 ± 0.5 (11) | 85.6 ± 20 (10)       |
| HMM-1P    | 10.6 ± 0.6 (9)  | 110 ± 22 (9)        |
for the longer times (see Fig. 3c). This protocol was repeated between 5 and 35 times per HMM ensemble tested. Displacement data were recorded, and displacement traces at a given load were fit to a least-square regression line to estimate velocity at that load (see Fig. 3b). Data were collected from multiple surface beads (8 for HMM-2P and HMM-1P) and from multiple flow cells (5 for HMM-2P, 3 for HMM-1P). Velocity data for a given load for all experiments were compiled and then fit to the Hill force-velocity \((F-V)\) relation (32) with the fitting parameters being the maximum isometric force \(F_0\) and constants \(a\) and \(b\),

\[
(F + a)(V + b) = (F_0 + a)b
\]

\[\text{(Eq. 2)}\]

**RESULTS**

**Single HMM Molecule Binding Events in the Optical Trap**—The mechanics and kinetics of unphosphorylated HMM, HMM-1P, and HMM-2P molecules were characterized at the single molecule level using the optical trap. Representative raw data traces are shown in Fig. 1 for the HMM-1P and -2P constructs. No data traces are shown for the unphosphorylated HMM as mechanical activity could not be recorded from this construct even when the surface density was increased 100-fold above that required to operate in the single molecule regime. We, therefore, conclude that the unphosphorylated HMM is completely inhibited, and no further description of this species will be presented. In contrast, HMM-1P and -2P binding and subsequent displacements were identified by a reduction in the noise (i.e. variance) and a shift in mean value of the displacement-time trace. These data were analyzed using mean-variance analysis (Fig. 1, right) to estimate \(d\) and \(t_{on}\) (see “Experimental Procedures”).

Both mean step size and attachment time for the HMM-1P were not significantly different from those of the HMM-2P (see Table 1) and compare well with previous measurements for smooth HMM-2P at 10 \(\mu\)M ATP (26, 27). These data show that HMM-1P and HMM-2P molecules cannot be distinguished mechanically or kinetically by this assay, noting that only one head of the molecule generates motion at any given time (see “Discussion”).

**Actin Filament Speed and Duty Ratio Estimates**—Actin filament speed in the motility assay over a saturated HMM surface (i.e. 100 \(\mu\)g/ml NAV and HMM, see below) was not statistically different for HMM-1P (0.59 \(\pm\) 0.10 \(\mu\)m/s, \(n = 61\) filaments) and HMM-2P (0.67 \(\pm\) 0.10 \(\mu\)m/s, \(n = 46\) filaments), whereas no motility was observed with the unphosphorylated HMM, as shown previously (1). Actin filament speed is determined by both the myosin head duty ratio, \(\alpha\), and the number of myosin heads available to interact with the actin filament (see Equation 1). Thus, for low duty ratio motors the speed decreases as the number of heads capable of interacting with the actin filament is reduced (30, 31) (see Fig. 2, c–e).

To estimate the duty ratio of HMM-1P and -2P, we analyzed the relationship between an actin filament speed and its length (Fig. 2, c–e), where the actin filament length dictates the number of available heads based on the HMM surface density (31). The HMM surface density was controlled by specific attachment of the HMM-1P and HMM-2P to the NAV-coated surface through their common C-terminal biotin tags. By limiting the NAV concentration, we could vary the head density identically for both constructs. The HMM surface density was characterized by measuring the extent of fluorescent actin bound to HMM in the absence of ATP as the NAV surface concentration was varied (Fig. 2, a and b, and supplemental Fig. S1). Based on these data, we estimate that using 100 \(\mu\)g/ml biotinated-HMM will saturate the flow cell surface at NAV concentrations >50 \(\mu\)g/ml, whereas at NAV concentrations <50 \(\mu\)g/ml, the HMM surface densities are less than saturating and proportional to NAV concentration.

We initially performed this assay at 20 \(\mu\)g/ml NAV. As expected for a low duty ratio motor, actin filament speeds for
both HMM-1P and -2P were slower for short actin filaments, where the number of available heads is limiting. Interestingly, at long actin filament lengths where the number of heads would be sufficient to generate maximum actin filament speeds, the HMM-1P generated half the maximum speed of the HMM-2P (Fig. 2c), in contrast to speeds that were equivalent for the two constructs at saturating HMM surface densities (see above). This result suggests that for these long actin filament lengths, the surface spacing between HMM-1P was sufficiently large so that HMM-generated displacements were not efficiently transmitted along the actin filament (see “Experimental Procedures” and Ref. 31).

From the best fit of Equation 1 to the actin filament speed versus filament length relationship (Fig. 2c), the duty ratio can be estimated, but its absolute value is dependent on the HMM surface density. Although the relative HMM surface densities are known (see Fig. 2b), we assumed that 34 HMM-2P heads/μm actin filament length are available so that the α value for HMM-2P equaled the previously characterized 4% duty ratio for phosphorylated smooth muscle myosin (31). This resulted in an α of 2.0 ± 0.8% for the HMM-1P, approximately half that of HMM-2P (Fig. 2c).

If the HMM-1P duty ratio is approximately half that of the HMM-2P, then either doubling the HMM-1P surface density relative to the HMM-2P or reducing the HMM-2P surface density relative to the HMM-1P should result in similar speed versus filament length relationships for the two constructs. This was observed as shown in Fig. 2, d and e. HMM density was varied by changing the NAV concentration as indicated (Fig. 2, d and e). By using all of the HMM-1P and -2P relationships under the various surface densities studied, the data could be globally fit, resulting in the HMM-1P having 0.6 ± 0.2 times the duty ratio of HMM-2P. It is worth noting that although the absolute α values are dependent on the HMM surface density, the ratio of the α values is not, provided that the duty ratios are small compared with 1 (see the supplementary material). Thus, the HMM-1P duty ratio is 60% that of HMM-2P.

**Force-Velocity Measurements**

Using the force clamp optical trap, velocities of a bead-actin-bead assembly over a population of HMM molecules were recorded at different loads (Fig. 3). These data were used to construct force-velocity curves for HMM-1P and -2P. For the HMM-2P, the velocity decreased with increasing load (21) and approached stall at the 10-pN loads applied (Fig. 3c). When using the same HMM-1P surface density, the HMM-1P ensemble generated insufficient force to translocate the bead-actin-bead assembly in this assay. However, when the effective HMM-1P density was increased 1.6-fold to the maximum possible under these experimental conditions (see “Experimental Procedures” and supplemental Fig. S2), force-velocity data were then obtained for the HMM-1P (Fig. 3, b and e).
of the HMM-2P construct at all forces. These force-velocity data suggest that the force-generating capacity of the HMM-1P is half that of HMM-2P (see the inset in Fig. 3c), possibly due to its lower duty ratio (see above).

**FIGURE 3.** Force-velocity protocol and relationships for HMM-1P and -2P. a, protocol for obtaining the velocity response of an ensemble of HMM molecules to various loads. A staircase of various loads (1–10 pN) was applied in the optical trap to an actin filament in contact with an HMM ensemble (see “Experimental Procedures” for details). The resultant displacement of the actin filament at these varying loads is depicted below the load staircase with the vertical lines demarcating the period over which a given load was applied. b, velocity obtained from displacement versus time traces under various loads. Examples of individual displacement traces in response to a fixed load as indicated to the right of each trace. Such traces were obtained from a staircase load protocol as in panel a. For each load the velocity was determined by fitting a linear regression (solid line) through the displacement-time trace. Note that the slope (i.e. velocity) decreases as the load increases. c, HMM-1P and -2P force-velocity relationships. Applied force and velocity data as measured in panels a and b for HMM-2P (filled circles) and HMM-1P (open circles) are shown. Data points are the mean ± S.E. from eight experiments for each construct resulting in between 11 and 67 individual velocity measurements per data point in the plot. For a given surface bead and the HMM ensemble sampled from it, up to 35 staircase protocols were applied. For the HMM-2P, the solid curve is the fit to the Hill force-velocity relationship (see “Experimental Procedures” and Equation 2) with the following parameters: $F_0 = 14.6$ pN; $a/F_0 = 0.35$; $b = 0.03 \mu$m/s. Because the HMM-1P data were obtained with 1.6 times more molecules than HMM-2P, the HMM-1P force data are normalized to the effective HMM surface density by dividing the HMM-1P forces by 1.6. Once normalized, the HMM-1P data were plotted as shown with the force-velocity fit having the following parameters: $F_0 = 11.1$ pN; $a/F_0 = 0.10$; $b = 0.01 \mu$m/s. The force-velocity for the HMM-1P is shifted to the left of the HMM-2P data. The inset takes the force-velocity data as plotted and divides the force for each point by the number of phosphorylated RLC. Therefore, the HMM-2P force values are divided by 2. Interestingly, by normalizing the data in this way, the HMM-2P data overlays the HMM-1P, suggesting that HMM-1P generates half the force of HMM-2P with the fit parameters $F_0 = 9.3$ pN; $a/F_0 = 0.16$; $b = 0.02 \mu$m/s.

**DISCUSSION**

Here we use both single molecule techniques to measure unitary displacements and attachment times and small ensemble measurements of HMM to measure force at different loads to more fully understand the force and motion-generating capabilities of smooth muscle HMM that has only one of its two RLCs phosphorylated. This species is expected to play an important role during smooth muscle activation as well as relaxation. It is well established that unphosphorylated smooth muscle myosin is “off,” which requires the presence of two heads (33–36) that presumably involves an asymmetric inter-head interaction (7), rendering both heads incapable of ATPase activity and motion generation. Phosphorylation of both RLCs disrupts this interaction so that each head can associate with actin to generate force and motion. Given this structural view of regulation, the question becomes whether phosphorylation of one RLC disrupts the asymmetric head interactions, and if it does, are the individual heads associated with the phosphorylated and unphosphorylated RLCs in the HMM-1P molecule mechanically equivalent?

**Single Molecule Data: HMM-2P Versus HMM-1P**—The optical trap provides a sensitive measure of myosin inherent displacement generation ($d$) and the time that the motor remains attached to actin after the power-stroke ($t_{on}$). When characterizing unphosphorylated HMM, the lack of any mechanical activity confirms that this molecule adopts the inhibited conformation. In contrast, both the power-stroke displacement ($d$) and strong binding lifetime ($t_{on}$) of HMM-1P were identical to that of HMM-2P, consistent with the actin filament speeds for these constructs being similar in the motility assay (1). We previously demonstrated that displacement events in the optical trap are generated by only one head of myosin (26, 37). For example, a heterodimeric smooth muscle HMM construct having one wild type and one obligatory weak binding mutant head generates $d$ and $t_{on}$ identical to that of HMM-2P (26). Thus, the present results cannot determine whether one or both heads of HMM-1P are mechanically competent but at a minimum confirms that HMM-1P is more like the HMM-2P rather than the unphosphorylated species.

A recent optical trap study by Yanagida and co-workers (20) characterized the step displacement and strong binding lifetime of unphosphorylated and single- and double-phosphorylated myosin. Surprisingly, the unphosphorylated myosin in their study bound strongly to actin filaments with the majority of events having lifetimes of ~1 s. These data are contrary to biochemical studies, which characterize the unphosphorylated, inhibited state of myosin as having only weak binding capacity (2, 3), as suggested by our own optical trap data with unphosphorylated HMM. It is possible that the biochemical conditions used by Tanaka et al. (20) to exchange RLC in their preparation may have resulted in a small fraction of modified, unregulated myosin capable of binding actin. If so, this might explain their observation that single-phosphorylated myosin has both short lifetimes characteristic of their double-phosphorylated species and a fraction of long lifetimes equivalent to their unphosphorylated myosin (20). The molecular mechanics of their single-phosphorylated myosin could sim-
Single-phosphorylated Smooth Muscle HMM

...ply be a mixture of single-phosphorylated myosin that, as in our study, is indistinguishable from the double-phosphorylated species and a modified unregulated component to account for the long-lived lifetime component.

Duty Ratio and Force-Velocity—Each HMM-1P head has approximately half the duty ratio of HMM-2P (Fig. 2). Because the duty ratio estimates the fraction of the ATPase cycle ($t_{on} + t_{off}$) that the head is strongly bound ($t_{on}$) to actin (i.e. $\alpha = t_{on}/(t_{on} + t_{off})$), a change in $\alpha$ could indicate an alteration in $t_{on}$ and/or $t_{off}$. For low duty ratio motors, $t_{on} \ll t_{off}$ so that $\alpha \approx t_{on}/t_{off}$. The similarity in both actin filament speed for HMM-1P and -2P at saturating HMM surface densities (see “Results” and in Rovner et al. (1)), which is limited by $t_{on}$ and the similarity in $t_{on}$ measured directly in the optical trap at low ATP suggest that $t_{off}$ for HMM-1P is twice that of HMM-2P to account for the lower duty ratio.

HMM-1P also generates approximately half the force of HMM-2P when the force-velocity relationships are normalized on a per head basis (see Fig. 3c). The force ($F$) produced by a population of $n$ HMM heads is related to the time averaged force generation ($F_{avg}$) of a single head ($F = nF_{avg}$). Because an HMM head can only support force while strongly bound, $F_{avg}$ is the product of its force while strongly bound ($F_{uni}$) and its duty ratio under loaded conditions ($\alpha_{Load}$), i.e. $F_{avg} = \alpha_{Load}F_{uni}$. If we assume that $F_{uni}$ is the same because the step sizes for HMM-1P and -2P are identical, then the reduced force under load ($F = n\alpha_{Load}F_{uni}$) associated with phosphorylating a single RLC could result from alterations to the kinetics of the HMM-1P head (i.e. a change in $\alpha_{Load}$) or to a reduction in the effective number of mechanically competent heads (i.e. $n$).

Modeling the Functional Consequences of HMM-1P—Numerous protein biochemical, structural, and mutational studies have focused on the structural elements that are essential to adopt the off state of smooth muscle myosin (2). Minimal requirements include that the molecule must form a stable double-headed structure with a sufficient length of rod that is flexible enough to allow the required head-head interactions to occur. This would explain why single-headed myosin, the S1 head of myosin, and constructs with short rods that have been artificially dimerized with a leucine zipper are active when unphosphorylated (38). Mutations to regions involved in stabilizing the off state also disrupt the ability to adopt the inhibited conformation. Altering charged residues in surface loop 2, which is part of the actin binding domain, lessen the ability of the blocked head to interact with the converter domain of the free head and, thus, activates the molecule (39).

Our data suggest that phosphorylation of one RLC is also sufficient to disrupt these interactions as HMM-1P generates substantial force and motion. If we assume that the inhibited and active states of smooth muscle myosin are in equilibrium and that phosphorylating both RLCs shifts this equilibrium toward the active state (Fig. 4), it is then possible that phosphorylation of only one RLC may also bias this equilibrium toward the active state. However, not knowing the extent to which HMM-1P shifts this equilibrium, two mechanisms are possible (see Fig. 4); 1) the equilibrium is such that all HMM-1P molecules are active, but the heads differ in their hydrolytic and mechanical properties; 2) phosphorylation of a single RLC cannot fully overcome the interactions between the two heads, so that the equilibrium constant between the inhibited and active states is $\sim 1$ under our experimental conditions, resulting in effectively half of the HMM-1P population being active and indistinguishable from HMM-2P.

If phosphorylation of one RLC shifts the equilibrium so that all HMM-1P molecules are active (Mechanism 1), then the kinetics for each molecule must differ to account for the lower apparent duty ratio and force observed here and the lower actin-activated steady state and single turnover ATPase rates of HMM-1P compared with HMM-2P reported previously (1, 19). Because each HMM-1P has two heads, it is possible that the two heads have different kinetics (i.e. attachment rates) depending on the RLC phosphorylation state of that head. For example, mutations to or deletion of the RLC can have significant effects on myosin force and motion generation (40). Thus, the effective kinetics of an HMM-1P molecule would be determined by the combined kinetics of the individual heads.

However, an alternate mechanism (Mechanism 2) is that HMM-1P molecules are in rapid equilibrium between the active and inhibited state, with an equilibrium constant of $\sim 1$ under the conditions of our experiments. If the active HMM-1P molecule is indistinguishable from an HMM-2P molecule (as in the optical trap), then this simple mechanism effectively reduces the number of active molecules at any point in time by approximately half. This would account for the apparent reduction in duty ratio and force data, as the effective HMM-1P surface density would be decreased.

FIGURE 4. Proposed mechanisms for the effect of regulatory light chain phosphorylation on smooth muscle HMM activity. Unphosphorylated HMM activity is inhibited by an asymmetric interaction between the blocked and free head. Upon phosphorylation of both regulatory light chains (red), the head-head interactions are disrupted, shifting the equilibrium (100% and bold arrow) to the fully active molecule, where each head is enzymatically and mechanically competent as indicated by a 1. The results presented here suggest two alternate mechanisms for the effect of phosphorylating a single regulatory light chain (HMM-1P). In Mechanism 1, as for HMM-2P, the head-head interactions are disrupted, and the equilibrium is shifted to an active molecule, where at least one head is enzymatically and mechanically competent to its fullest extent, whereas the other head may have altered kinetics relative to a normal head affecting its hydrolytic and mechanical properties. Alternately, in Mechanism 2, phosphorylating a single regulatory light chain cannot fully disrupt the head-head interactions so that the HMM-1P exists in an equilibrium where the probability of being in the inhibited and active conformations is nearly equal (i.e. 60% active and 40% inhibited). In the active state, the HMM-1P molecule is indistinguishable from an active HMM-2P molecule. The 60% reflects the reduction in the duty ratio for the HMM-1P compared with the HMM-2P (Fig. 2).
It should be noted that elements of both mechanisms can also co-exist. For example, there may be an equilibrium between the active and inhibited states, but there may also be differences between the phosphorylated and unphosphorylated head in the active species, although to a lesser extent than depicted in Mechanism 1.

Conclusions—The present results agree with previous studies (19, 20) in that single-phosphorylated smooth muscle myosin and HMM molecules are active species. This result is relevant for smooth muscle physiology in that single-phosphorylated myosin must exist during activation and, more importantly, during sustained periods of force maintenance. Smooth muscle tissue has the ability to maintain force with little energy expenditure even though the level of RLC phosphorylation is reduced compared with that at the peak of activation. Various models have been proposed to explain the ability of smooth muscle tissue to maintain force with little energy expenditure even though the level of RLC phosphorylation is still debated, with our study showing it to be because our protein expression system does not require the exchange (19, 20).

The level of hydrolytic and mechanical capacity of HMM-1P is still debated, with our study showing it to be because our protein expression system does not require the exchange (19, 20). It is possible that the methods for preparing HMM-1P contribute to these experimental differences, because our protein expression system does not require the additional biochemical procedures required for RLC removal or exchange (19, 20).

With regard to tonic force generation in smooth muscle tissue, only the present study characterized the force generation of the single-phosphorylated species (Fig. 3). Knowing that the HMM-1P, having one of its RLCs unphosphorylated, is still capable of generating at least half the force of the fully phosphorylated HMM, this species will be an important contributor to the capacity of smooth muscle tissue to maintain force despite a reduced level of RLC phosphorylation. Although we cannot distinguish between the two mechanisms for HMM-1P functionality proposed here (see Fig. 4), future biophysical and modeling studies may help distinguish between these proposed mechanisms.

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