Cooperative attachment of cross bridges predicts regulation of smooth muscle force by myosin phosphorylation

Christopher M. Rembold,1,2 Robert L. Wardle,2 Christopher J. Wingard,2 Timothy W. Batts,1,2 Elaine F. Etter,1 and Richard A. Murphy2

1Cardiovascular Division, Department of Internal Medicine, and 2Department of Molecular Physiology and Cellular Biophysics, University of Virginia, Charlottesville, Virginia 22908

Submitted 10 February 2004; accepted in final form 13 May 2004

First published May 19, 2004; 10.1152/ajpcell.00082.2004.

Cooperative attachment of cross bridges predicts regulation of smooth muscle force by myosin phosphorylation. Am J Physiol Cell Physiol 287: C594–C602, 2004.—Serine 19 phosphorylation of the myosin regulatory light chain (MRLC) appears to be the primary determinant of smooth muscle force development. The relationship between MRLC phosphorylation and force is nonlinear, showing that phosphorylation is not a simple switch regulating the number of cycling cross bridges. We reexamined the MRLC phosphorylation-force relationship in slow, tonic swine carotid media; fast, phasic rabbit urinary bladder detrusor; and very fast, tonic rat anococcygeus. We found a sigmoidal dependence of force on MRLC phosphorylation in all three tissues with a threshold for force development of ~0.15 mol P/mol MRLC. This behavior suggests that force is regulated in a highly cooperative manner. We then determined whether a model that employs both the latch-bridge hypothesis and cooperative activation could reproduce the relationship between Ser19-MRLC phosphorylation and force without the need for a second regulatory system. We based this model on skeletal muscle in which attached cross bridges cooperatively activate thin filaments to facilitate cross-bridge attachment. We found that such a model describes both the steady-state and time-course relationship between Ser19-MRLC phosphorylation and force. The model required both cooperative activation and latch-bridge formation to predict force. The best fit of the model occurred when binding of a cross bridge cooperatively activated seven myosin binding sites on the thin filament. This result suggests cooperative mechanisms analogous to skeletal muscle that will require testing.

SKELETAL MUSCLE CONTRACTION is regulated at the thin filament: Ca2+ binds to troponin C (TnC), inducing a conformational change in troponin I (TnI) and tropomyosin (TM) that uncovers myosin binding sites on actin reviewed by Chalovich (4) and Gordon et al. (12). Constitutively activated myosin cross bridges are then free to bind to the thin filament, producing cross-bridge cycling and force generation. The implication of this regulatory system is that the amount of force depends on the number of free myosin binding sites on actin. Therefore, force depends on the amount of Ca2+ bound to TnC. The relationship between free myoplasmic Ca2+ concentration ([Ca2+]i) and force is sigmoidal. The velocity of unloaded shortening (V0) is the same at both high and low numbers of free myosin binding sites on actin. Reduction in force (relaxation) occurs when reductions in [Ca2+]i cause dissociation of Ca2+ from TnC, permitting TnI and TM to block binding of myosin cross bridges. If [ATP] is reduced, some myosin cross bridges remain bound to actin (these are “rigor” bonds), preventing TnI and TM from inhibiting the region of seven actins spanned by TM. This is a form of cooperative activation by which myosin binding can activate actin and induce further myosin binding.

There is no troponin in vertebrate smooth muscle (reviewed by Sellers and Adelstein (35)). Ca2+ binds to calmodulin (CaM) and activates myosin light chain kinase (MLCK) (16). MLCK regulates myosin by phosphorylating the 20-kDa myosin regulatory light chain (MRLC) at Ser19 (41). Ser19-MRLC phosphorylation increases myosin’s actin-activated ATPase activity by approximately 1,000-fold (28). Once phosphorylated, the myosin cross bridge can bind to actin, generating force by cross-bridge cycling. This thick filament regulatory system significantly differs from the thin filament system in skeletal muscle: J V0 is variable and is linearly dependent on Ser19-MRLC phosphorylation in the agonist-activated or K+ -depolarized swine carotid (7), and 2) the relationship between MRLC phosphorylation and force is not linear (29). The latch-bridge hypothesis sought to explain these findings by positing that an attached phosphorylated cross bridge (see Fig. 5, top) could be dephosphorylated to form an attached dephosphorylated cross bridge (a “latch bridge”) that could maintain force because it would have a slower detachment rate than a phosphorylated cross bridge (13). The presence of force-maintaining latch bridges could explain the variable V0 in smooth muscle (14).

A quantitative model of the latch-bridge hypothesis, given several key assumptions, predicted the nonlinear dependence of force on Ser19-MRLC phosphorylation (13, 31). One of these key assumptions was that basal MRLC phosphorylation values (~0.05–0.10 mol P/mol MRLC) were artificially elevated. It was hypothesized that superficial damaged cells had high [Ca2+]i, and, therefore, very high MRLC phosphorylation that contaminated the very low MRLC in the remaining

Address for reprint requests and other correspondence: C. M. Rembold, Box 801395, Cardiovascular Division, Univ. of Virginia Health System, Charlottesville, VA 22908-1395 (E-mail: crembold@virginia.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
tissue (13). This assumption was necessary because the quantitative latch-bridge model predicts that small increases in Ser\(^{19}\)-MRLC phosphorylation above zero should be associated with significant increases in force (13).

Unfortunately, MRLC phosphorylation in these studies was quantitated as a simple ratio of intensity from Western blots or Coomassie-stained gels. Simple ratios are subject to both offset and saturation errors that systematically underestimate low levels of phosphorylation (45). It is now apparent that simple intensity ratios systematically underestimated low values of MRLC phosphorylation. The first goal of the present study was to repeat analysis of the relationship between MRLC phosphorylation and force in several types of smooth muscle with a dilution immunoassay method that minimizes errors that occur with a simple ratio method (45). As is apparent in our data, there is a steep dependence of force on MRLC phosphorylation with a threshold phosphorylation value of some 0.15 mol P/mol MRLC. This threshold phosphorylation suggests that the latch-bridge model (which predicts no threshold phosphorylation value) is incomplete.

A second key assumption of the latch-bridge model is that it does not incorporate cooperative attachment of cross bridges. Cooperativity has been reported in smooth muscle (1, 15, 18, 19, 22, 39, 44). Smooth muscle actin filaments contain a smooth muscle isoform of TM that could be involved in a cooperative mechanism of activation. Published data show that the latch-bridge model (which predicts no threshold phosphorylation value) is incomplete.

The alternative is that Ser\(^{19}\)-MRLC phosphorylation, through cross-bridge attachment, regulates force by both the latch-bridge mechanism and cooperative activation. The second goal of this study was to determine whether a model that employs both the latch-bridge hypothesis and cooperative activation could reproduce the observed relationship between Ser\(^{19}\)-MRLC phosphorylation and force without the need for a second regulatory system.

**METHODS**

**Tissues.** Swine carotid arteries obtained from a slaughterhouse were transported to the laboratory on ice in physiological saline solution (PSS) containing (in mM) 140 NaCl, 4.7 KCl, 1.2 NaHPO\(_4\), 1.6 CaCl\(_2\), 1.2 MgCl\(_2\), 5.6 d-glucose, 2.0 3-(N-morpholino)propanesulfonic acid buffer (pH 7.4 at 4 or 37°C), and 0.02 ethylenediamine-N,N\(^{-}\)-tetraacetic acid (EDTA) to chelate trace heavy metal ions. Eight to sixteen medial rings free from adventitia and endothelium were prepared from individual arteries (49). Approximately 2-mm-wide rings were mounted on two posts attached to a micrometer and a Grass FT 03 force transducer, respectively. The rings were equilibrated and adjusted to the optimum length for force development (L\(_o\)) in organ baths held at 37°C and gassed with compressed air. The conditioning contractions for which optimum length was established were elicited by 109 mM K\(^{+}\) depolarization (KCl substituted stoichiometrically for NaCl in PSS). Forces were normalized to the maximum value elicited upon depolarization. Data were discarded if this maximum force was <1 × 10\(^{-2}\) N/m\(^2\).

Male New Zealand White rabbits (2.3–2.7 kg) were euthanized by halothane inhalation according to an Institutional Animal Care and Use Committee-approved protocol. The urinary bladder was isolated at 4°C in a bicarbonate-buffered Krebs solution containing (in mM) 117.8 NaCl, 6.0 KCl, 24.3 NaHCO\(_3\), 1.2 NaH\(_2\)PO\(_4\), 1.6 CaCl\(_2\), 1.2 MgSO\(_4\), 5.6 d-glucose, and 0.026 Na\(_2\)-EDTA saturated with 95% O\(_2\)-5% CO\(_2\). An incision was made from the bladder neck up to the urethra and following either the dorsal or ventral vascular lamina. The bladder was pinned out with the mucosa facing down. This protocol causes the ridges to form from which strips were dissected from the aboluminal surface. Histological examination showed that the cells were aligned with the longitudinal axis of the preparation in which length and force were measured (not shown). The bladder strips were tied to the two posts on the apparatus with silk sutures, and the length was incrementally increased until a constant force of 1 g was maintained, approximating L\(_o\). The preparations responded with sustained contractions when exposed to 3 μM carbachol. K\(^{+}\) depolarization elicited transient contractions diagnostic of a phasic smooth muscle (38). Tissues exhibiting spontaneous oscillatory activity were excluded from the analysis.

Sprague-Dawley rats (200–350 g) of either sex were euthanized by halothane overdose and subsequently exsanguinated to provide a blood-free dissection field. Two anococcygeus muscles were dissected from each rat (46). Tissues were bathed in a Krebs solution gassed with 95% O\(_2\)-5% CO\(_2\) containing (in mM) 118.0 NaCl, 4.75 KCl, 2.54 CaCl\(_2\), 1.18 MgSO\(_4\), 1.18 KH\(_2\)PO\(_4\), 24.8 NaHCO\(_3\), and 10.0 d-glucose (pH 7.36 at 37°C). Tissues (2–8 mg) were stretched to 1 g of sustained force, equilibrated for 1 h, and then contracted for 10 min with 109 mM K\(^{+}\)-containing Krebs in the presence of 100 μM N\(^{G}\)-nitro-L-arginine, a nitric oxide synthase inhibitor.

Details of experimental protocols varied among smooth muscle types and are indicated in the figure legends. The protocols and the constituents of the physiological saline differed in these preparations because we sought to optimize consistent responses while minimizing resting tone and spontaneous activity.

**MRLC phosphorylation.** Arterial medial rings, bladder strips, or anococcygeus muscles were frozen in 20 ml of acetone cooled with 20 ml of crushed dry ice. They were then slowly (2.5 h) thawed to room temperature, to dehydrate the tissues, and were then air dried and weighed. The dry samples were homogenized in ground-glass tissue homogenizers on ice in 1% (wt/vol) sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, and 20 mM dithiothreitol (0.22 ml/mg tissue dry wt) and were then centrifuged at 14,000 g for 10 min. Trichloroacetic acid was not included because it did not alter MRLC phosphorylation estimates. Serial dilutions (1:2, 1:4, 1:8, 1:16, and 1:32) of homogenates in homogenization buffer were loaded onto 12% acrylamide/glycerol-urea slab gels for isoelectric focusing at 250 V overnight on a pH 4–6.5 gradient (45). The separated proteins were transferred to nitrocellulose membranes by wet electrophobting and immunolabeled with rabbit polyclonal anti-MRLC antibody. The blots were then developed with secondary horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad) and detected with amplified Opti-4CN kits (Bio-Rad) or enhanced chemiluminescence substrate (Amersham).

Air-dried blots were scanned (HP ScanJet 6200C) next to a reflective gray scale calibration tablet (Kodak). The resulting images were digitized (UN-SCAN-IT software, Silk Scientific, and the background-subtracted integrated pixel intensity of phosphorylated and dephosphorylated MRLC images was measured. X-ray film exposed to enhanced chemiluminescence was scanned using a Molecular Dynamics laser densitometer and analyzed with NIH Image software (14). The relative protein content was estimated by assuming that antibody binding was the same for phospho- and dephospho-MRLC (45).

We analyzed MRLC phosphorylation with a multiple dilution assay because simple ratio assays are subject to both offset and saturation...
errors (45). To understand this effect, consider a tissue with MRLC phosphorylation of 0.20 mol P/mol MRLC. We typically find immuno-nostaining intensities for unphosphorylated light chains to have values of 50, 50, 25, 12, and 2 for full, 1:2, 1:4, 1:8, and 1:16 dilutions, respectively. For phosphorylated light chains, the values are 25, 12, 2, 0, and 0, respectively. It is apparent that the full-strength unphosphorylated intensity is saturated because there is no change from the full to 1:2 dilution. Therefore, if we were to do a simple ratio of the full strength, the MRLC phosphorylation would be 25/25 (50%), or 33%. It is also apparent that there is signal dropout at intensities under 12.

Therefore, if we were to do a simple ratio of the 1:4 dilution, the MRLC phosphorylation would be 2/2 (50%), or 7%. The actual phosphorylation is best measured by interpolation as detailed previously (45) or by comparison of similar intensities, taking dilution into account. For example, intensity was similar with unphosphorylated light chains at a 1:4 dilution and phosphorylated light chains at full strength; therefore, phosphorylation is 25/(25 + (25 × 4)), or 20%.

Models. Models of smooth muscle contraction were performed on an IBM compatible personal computer in a manner similar to that performed previously (31, 32). The differential equations (Eqs. 6–15, see RESULTS) were solved with a fifth-order Runge-Kutta algorithm purchased from Promath (Hammerly Computer Services, Laurel, MD) in compiled QuickBASIC 4.5 (Microsoft).

For modeling, we used a detailed time course of electrically stimulated swine carotid according to the method of Singer et al. (36). This time-course study employed the simple ratio methods for measuring Ser19-MRLC phosphorylation. Fortunately, stimulated values of Ser19-MRLC phosphorylation reported by Singer et al. ranged between 0.38 and 0.60 mol P/mol MRLC, values that do not require dilution for accurate measurement (45). Therefore, these “older” single dilution measurements of Ser19-MRLC phosphorylation should be comparable with the newer dilution assay.

RESULTS

Steady-state dependence of force on MRLC phosphorylation. Figure 1 shows the relationship between MRLC phosphorylation and force in swine carotid artery when MRLC phosphorylation was estimated using a simple ratio without dilution. In this data set, considerable effort was taken to adjust the volume of the tissue extract on the gels to facilitate detection of low levels of phosphorylation. There was a unique relationship between MRLC phosphorylation and force in tissues activated with histamine or K+ depolarization. With this single-ratio method, increases in force were noted with MRLC phosphorylation in a similar manner in urogenital tissues. Figure 3 shows data from rabbit urinary bladder detrusor strips as an example of a fast, phasic smooth muscle. There was a steep dependence of force on MRLC phosphorylation with a threshold phosphorylation value of ~0.15 mol P/mol MRLC. A similar dependence and threshold were observed in limited studies of the rat anococcygeus smooth muscle (Fig. 4). The anococcygeus smooth muscle is an unusual example of a physiologically tonic muscle with a high V0 for a given level of MRLC phosphorylation (11, 46).

We were unable to obtain higher steady-state levels of phosphorylation in the detrusor and anococcygeus muscles, so statistically meaningful Hill plots and coefficients were not obtained for comparison with the carotid.

Development of the model. Our second goal was to build a biologically based model of force regulation in smooth muscle to evaluate whether cooperative cross-bridge attachment could explain the observed dependence of force on MRLC phosphorylation (Figs. 2–4). The model of cross-bridge activation is illustrated in Fig. 5, top. Previously, we (32) modeled direct attachment of dephosphorylated cross bridges (a K0 attachment rate with M → AM, where M represents detached myosin and AM represents dephosphorylated myosin attached to actin; i.e., a latch bridge) without showing an improvement in prediction of the dependence of force on MRLC phosphorylation over...
that predicted when $K_a = 0$. We therefore decided to base smooth muscle cooperativity on the skeletal muscle paradigm, with cooperativity changing the attachment rate of phosphorylated cross bridges ($K_i$ with $M_p \rightarrow A_{M_p}$, where $M_p$ represents phosphorylated myosin not attached to actin and $A_{M_p}$ represents phosphorylated myosin attached to actin).

Our model of cooperativity requires knowledge of the number of myosin binding sites on actin and the number of myosin cross bridge heads present in a typical smooth muscle cell. Assuming a smooth muscle cell is a box, with dimensions $5 \times 5 \times 40 \mu m$ long, the volume is $10^{-15} m^3$; i.e., $10^{-12}$ liter, or 1 pl. Murphy et al. (26) found that the myosin content of swine carotid arterial smooth muscle was 13 g/kg. Given a density of 1.05 kg/l, this calculates to 13 g/kg. The molecular mass of myosin is 235 kDa per head, so $[\text{myosin heads}] = 58 \mu M$. Therefore, for a 1-pl cell, there would be $58 \mu M \times 1 \times 6.02 \times 10^{23}$ molecules ($\text{myosin heads/mole} = 3.5 \times 10^7$ myosin heads/cell). Murphy et al. also found that the actin content of swine carotid arterial smooth muscle was 42 g/kg. Given a density of 1.05 kg/l, this calculates to 44.1 g/l. The molecular mass of actin is 43 kDa, so $[\text{actin}] = 1.026 \mu M$. For a 1-pl cell, there would be $1.026 \mu M \times 1 \times 6.02 \times 10^{23}$ molecules/mole $= 6.17 \times 10^9$ actin molecules/cell. Overall, these calculations reveal $\approx 18$ actin binding sites per myosin head. These calculations were made before further modeling and can be considered "prespecified."

We assume that an attached cross bridge ($A_{M_p}$ or $A_{M}$) activates a specific number of myosin binding sites (i.e., actins) along a thin filament. For example, each myosin bound could activate the seven actins linked by one TM molecule in a manner analogous to skeletal muscle (12). We defined $C$ as the number of actins activated by each bound myosin. Given $C$ and the number of filamentous actin monomers present in the cell, we can calculate $A$ as the fraction of actin molecules activated by the binding of one cross bridge (assuming actin is primarily filamentous). We then can calculate the fraction of thin filaments (F) that will be activated by the binding of $n$ cross bridges. We assumed that cross bridges attach to sites on the thin filament randomly. This assumption is more plausible in smooth than in striated muscle, given the higher actin/myosin ratio (26). Therefore, each additional cross bridge head after the first can attach to either an “activated” or an “inactivated”
F_2 = 2A - A^2 \quad (1)

For the third cross-bridge binding, we need to add a third A multiplied by the fraction not already activated (1 minus the amount activated by the first 2 bindings), which is A + A(1 - A) + A[(1 - [A + A(1 - A)])], or

F_3 = 3A - 3A^2 + A^3 \quad (2)

The fourth term is A + A(1 - A) + A[1 - [A + A(1 - A)]) + A(1 - A[1 - [A + A(1 - A)])] or

F_4 = 4A - 6A^2 + 4A^3 - A^4 \quad (3)

and so on. The form of this series is the binomial expansion missing the first term of 1 and can be written

F_n = 1 - (1 - A)^n \quad (5)

where n is the number of bound cross bridges.

Because we want a cooperative binding model, we need to have two rate constants for binding of phosphorylated cross bridges: \( K_{3\text{ active}} \) is the rate constant for binding to an activated region, and \( K_{3\text{ inactive}} \) is the rate constant for binding to an inactivated region. The apparent rate constant \( K_3 \) (\( \text{Mp} \rightarrow \text{AMP} \); Fig. 5) is a function of \( K_{3\text{ active}} \), \( K_{3\text{ inactive}} \), and the fraction \( F_n \) (Eq. 9). This \( K_{3\text{ apparent}} \) can be inserted into the latch bridge model (Fig. 5) introduced by Hai and Murphy (13). The model has four differential equations with \( K_{3\text{ apparent}} \) calculated for each time period according to Eq. 6. The full set of equations is as follows:

\[
C = \text{no. of myosin binding sites (i.e., actin)}
\]

\[
A = C/\text{total no. of actin molecules} = C/6.17 \times 10^8
\]

\[
n = \text{total no. of myosin heads}
\]

\[
\times \text{fraction of attached cross bridges}
\]

\[
= 3.5 \times 10^7 \times ([\text{AMP}] + [\text{AM}])
\]

\[
F_n = 1 - (1 - A)^n
\]

(\( F_n \) was calculated as \( 1 - e^{n\ln(1 - A)} \) to reduce rounding errors.)

\[
K_{3\text{ apparent}} = F_n \times K_{3\text{ active}} + (1 - F_n) \times K_{3\text{ inactive}}
\]

\[
d[\text{M}] / dt = K_{3\text{ apparent}}[\text{M}] + K_{3\text{ active}}[\text{MP}] - K_{3\text{ inactive}}[\text{MM}]
\]

\[
d[\text{MP}] / dt = K_{3\text{ apparent}}[\text{MP}] + K_{3\text{ active}}[\text{AM}] - (K_{3\text{ inactive}})[\text{MM}]
\]

\[
d[\text{AMP}] / dt = K_{3\text{ apparent}}[\text{AMP}] + K_{3\text{ active}}[\text{AM}] - (K_{3\text{ inactive}})[\text{MM}]
\]

\[
\text{MRLC phosphorylation} = [\text{MP}] + [\text{AMP}]
\]

\[
\text{Force} = [\text{AMP}] + [\text{AM}]
\]

where \([\text{M}]\) is the fraction of unbound dephosphorylated myosin heads, \([\text{MP}]\) is the fraction of unbound phosphorylated myosin heads; \([\text{AMP}]\) is the fraction of bound phosphorylated myosin heads (i.e., force producing); and \([\text{AM}]\) is the fraction of bound dephosphorylated myosin heads (i.e., latch bridges that are force maintaining; see Fig. 5, top). The sum of \([\text{M}] + [\text{MP}] + [\text{AMP}] + [\text{AM}] = 1\), and rate constants are given in units of \( s^{-1}\). As in the prior model of Hai and Murphy (13), we used the number of attached cross bridges ([AMP] + [AM]) as an estimate of isometric force.

We started with rate constants (other than \( K_1 \)) equal to those employed by Hai and Murphy (13). \( K_1 \) and \( K_6 \) were set at variable levels to reflect variable MLCK activity. \( K_2 = K_5 = 0.5 \, s^{-1} \) for myosin phosphatase activity. \( K_4 = 0.1 \, s^{-1} \) for phosphorylated cross-bridge detachment (\( \text{AMP} \rightarrow \text{MP} \)). \( K_7 = \)
0.02 s\(^{-1}\) for dephosphorylated cross-bridge (latch bridge) detachment (AM \(\rightarrow\) M). We assumed that only phosphorylated cross bridges can bind to actin; therefore, there was no \(K_8\) (M \(\rightarrow\) AM).

**Fitting the model.** We varied \(k_{3\text{ active}}, k_{3\text{ inactive}},\) and \(C\) (the number of actin molecules activated per bound myosin head) to fit the observed steady-state relation between MRLC phosphorylation and force in swine carotid media. The best fit occurred when \(C = 7\), i.e., seven myosin binding sites were activated by binding of a cross bridge head. This is similar to the proposed mechanism for cooperativity in skeletal muscle (4, 12). We also found that the ratio of \(k_{3\text{ active}}\) to \(k_{3\text{ inactive}}\) should be high, paralleling the approximately 1,000-fold increase in myosin ATPase occurring with phosphorylation of MRLC on Ser\(^{19}\) (28). This approach fit the steady-state data.

Despite fitting the steady-state data, the model incorporating the Hai and Murphy (13) rate constants was too slow to fit the time-course data for electrically stimulated swine carotid media reported by Singer et al. (36). Therefore, \(k_2, k_4,\) and \(k_7\) were varied to fit both the steady-state and time-course data.

The best fit was with \(k_2 = 1.0 \text{ s}^{-1}, k_{3\text{ active}} = 3.0 \text{ s}^{-1}, \) \(k_{3\text{ inactive}} = 0.00001 \text{ s}^{-1}, k_4 = 0.4 \text{ s}^{-1}, k_5 = 0.1 \text{ s}^{-1},\) and \(C = 7\). The model fit the steady-state relationship between Ser\(^{19}\)-MRLC phosphorylation and force observed in swine carotid artery stimulated by various protocols (Fig. 5, **bottom**). As Ser\(^{19}\)-MRLC phosphorylation increased from 0 to \(\approx 0.15\) mol P/mol MRLC, there was an increase in predicted [Mp] and a decrease in [M] without significant increases in [AM], [AM], or force (Fig. 5, **middle**). Increases in Ser\(^{19}\)-MRLC phosphorylation above \(\approx 0.15\) mol P/mol MRLC induced a linear increase in [AM] and a more gradual increase in [Mp]. Increases in Ser\(^{19}\)-MRLC phosphorylation from \(\approx 0.15\) to \(\approx 0.30\) mol P/mol MRLC induced large increases in [AM], whereas values above \(\approx 0.30\) mol P/mol MRLC did not further increase [AM].

Figure 6 shows that the model fit the time-course data observed in electrically stimulated swine carotid artery by Singer et al. (36). Figure 6A shows how \(k_1\) (M \(\rightarrow\) Mp) was adjusted to fit the measured Ser\(^{19}\)-MRLC phosphorylation transient (B). The \(k_1\) transient increased estimated [Mp] quickly (Fig. 6C), but \(k_{3\text{ apparent}}\) (Mp \(\rightarrow\) AMp) increased more slowly, reflecting the time required for cooperative activation. As \(k_{3\text{ apparent}}\) increased, there was an increase in [AMp] followed by an increase in [AM]. After 5 s, [Mp] fell as Ser\(^{19}\)-MRLC phosphorylation fell and cross bridges became attached. The steady-state value of the \(k_{3\text{ apparent}}\) rate constant \((\approx 0.4)\) was similar to the value of the rate constant \(K_3\) published by Hai and Murphy (13). Figure 6D shows that the model was able to predict measured force.

**Characteristics of the model.** We evaluated the effect of changing the model’s constants as shown in Fig. 7. Decreasing \(C\), the number of myosin binding sites (i.e., actin activated per bound myosin), reduced force at all levels of Ser\(^{19}\)-MRLC phosphorylation (Fig. 7A). Increasing \(C\) increased force at all Ser\(^{19}\)-MRLC.

It is apparent that 10 sites clearly overestimated and 5 sites clearly underestimated the force. Changing \(k_{3\text{ inactive}}\) (the attachment rate of Mp \(\rightarrow\) AMp on inactive myosin binding sites) had little effect on force unless the value of \(k_{3\text{ inactive}}\) approached \(k_{3\text{ active}}\) (Fig. 7B). Modest decreases in \(k_{3\text{ active}}\) (the attachment rate of Mp \(\rightarrow\) AMp on active myosin binding sites) reduced force, whereas increases in \(k_{3\text{ active}}\) increased force (Fig. 7C). Decreases in \(k_2\) (myosin phosphatase activity) reduced force modestly, whereas increases in \(k_2\) increased force modestly (Fig. 7D). Increases in \(k_4\) (the detachment rate of AMp \(\rightarrow\) Mp) reduced force, whereas decreases in \(k_4\) increased force (Fig. 7E). Increases in \(k_7\) (the detachment rate of AM \(\rightarrow\) M) flattened the Ser\(^{19}\)-MRLC phosphorylation-force relationship (Fig. 7F). A very high value of \(k_7\) \((10 \text{ s}^{-1})\) resulted in no force, indicating that latch bridges (AM) were not required element in this model. Decreases in \(k_7\) increased force.

**DISCUSSION**

A sigmoidal dependence of force on MRLC phosphorylation was observed in slow, tonic swine carotid smooth muscle (Fig. 2), a relation previously described in permeabilized chicken gizzard (34) and in guinea pig longitudinal intestinal tissues (21). This relationship was well described with a Hill plot. The steep relationship with a threshold MRLC phosphorylation value was also observed in rabbit bladder smooth muscle (a phasic muscle with a high shortening velocity; Fig. 3) and in...
rat anococcygeus smooth muscle (a tonic muscle with a very high shortening velocity; Fig. 4). The results suggest that basal levels of MRLC phosphorylation in relaxed smooth muscle are significant: \( \sim 0.15 \text{ mol P}/\text{mol MRLC} \). Thus contraction elicited by Ser\(^{19}\)-MRLC phosphorylation requires a threshold value and is likely a highly cooperative process. In a fast, phasic bladder smooth muscle, this appears to approach the behavior of an on-off switch in the sense that small increases in suprathereshold MRLC phosphorylation elicit high active forces (Fig. 3).

Theories for regulation of cross-bridge attachment and cycling in smooth muscle must account for the sigmoidal dependence of force on MRLC phosphorylation shown in Figs. 2–4. The specific model proposed by Hai and Murphy (13) was generated to fit the quasi-hyperbolic dependence of force on MRLC phosphorylation originally observed (c.f., dashed curve in Fig. 1). This model clearly does not fit the data in Fig. 2–4, which show a threshold of \( \sim 0.15 \text{ mol P}/\text{mol MRLC} \).

The basic concepts of the latch-bridge hypothesis remain plausible. 1) Cross-bridge phosphorylation is obligatory for cross-bridge attachment to thin filaments. 2) The ensuing cycle of a phosphorylated cross bridge leading to shortening and force generation is relatively fast, depending on load and the myosin isoform. 3) Finally, dephosphorylation of an attached cross bridge by myosin light chain phosphatase slows detachment rates, lengthening the duty cycle, and allows force maintenance with reduced cross-bridge turnover and ATP consumption rates (latch) (40). However, a mechanism conferring cooperativity must be added.

A goal of this study was to determine whether a model that incorporates cooperative cross-bridge activation into the latch-bridge hypothesis could reproduce the relationship between Ser\(^{19}\)-MRLC phosphorylation and force in the swine carotid artery. We found that both cooperative activation and latch-bridge formation are required to fit the time-course and steady-state relationships between observed Ser\(^{19}\)-MRLC phosphorylation and force (Figs. 5 and 6). The original latch-bridge model alone required that basal levels of Ser\(^{19}\)-MRLC phosphorylation be subtracted to fit the data (13). Without this assumption, the latch-bridge model predicts that very small increases in Ser\(^{19}\)-MRLC phosphorylation would induce large increases in force.

The cooperative attachment model alone also did not predict the relationship between Ser\(^{19}\)-MRLC phosphorylation and...
force: Fig. 7F shows that a high value of $K_7$ causes latch bridges (AM) to quickly detach, forming M and resulting in very low predicted force. Therefore, neither the latch-bridge model alone nor a cooperative attachment model alone predicted force. Our model may explain why the relationship between MRLC phosphorylation and force was linear when skinned tissues were activated with adenosine 5'-O-(3-thiotriphosphate) (ATPγS) (17, 42). Because ATPγS forms non-hydrolyzable phosphorylated cross bridges, it would be expected that no latch bridges (AM) could form. This is the same result as if $K_7$ were increased to high values. Figure 7F shows a linear, right-shifted relationship between MRLC phosphorylation and force that is reminiscent of the prior ATPγS data.

Previously, Paul (27) created a model of smooth muscle contraction based on a high ratio of attachment ($K_A$) to detachment ($K_d$). We found that increasing $K_{3 \text{ active}}$ (Fig. 7C) or decreasing $K_2$ (Fig. 7E) cause a leftward shift the MRLC phosphorylation-force relationship, suggesting that such a high ratio cannot predict force in our model. The model described here generally predicts the relationship between steady-state Ser19-MRLC phosphorylation and force in a data set that was limited to Ser19-MRLC phosphorylation values <0.40 mol Pi/mol MRLC (Fig. 5). The model’s prediction of the Ser19-MRLC phosphorylation-force relationship was not quite as “steep” as the observed data. Changes in rate constants that made the relationship steeper slowed predicted time-course data (data not shown).

The finding that a model incorporating latch bridges and cooperative activation predicts the relationship between Ser19-MRLC phosphorylation and force does not rule out the possibility that there is a second system, beyond Ser19-MRLC phosphorylation, that regulates cooperativity. Potential candidates to regulate cooperativity may include caldesmon (5, 20, 23), calponin (10, 37, 47, 48), heat shock protein 20 (HSP20) (30); however, a regulatory role in contractile actin filament remodeling has been proposed (24). Interestingly, substantial changes in Ser19-MRLC phosphorylation without significant alteration in the steady-state MRLC phosphorylation-force relationship (unpublished data and Ref. 8). Intriguingly, substantial changes in MRLC phosphorylation activity (doubling or halving) did not substantially change force (Fig. 7D). This analysis suggests that changes in MRLC phosphorylation would primarily alter MRLC phosphorylation levels rather than force independent of MRLC phosphorylation. 2) We assumed that unphosphorylated myosin could not attach. We modeled this previously and found that it did not predict force well (32). 3) We assumed that phosphorylation of one myosin II head (cross bridge) did not alter the behavior of the other myosin II cross bridge. 4) We also assumed that there is no effect on force by dynamic assembly or disassembly of thick or thin filaments during contraction. Activation-induced assembly of thin filaments has been proposed (24). Significant changes in thick or thin filament structure could alter force generation but are beyond the scope of this model. 5) We did not include a series elastic component (SEC) in the model or consider agonist diffusion rates in fitting the time-course data. Addition of an SEC to the model would slow the estimated rate of force development but would not alter steady-state estimates. 6) Finally, all models are important only for the testable predictions made. We are in the process of testing the model’s energetic predictions and also predictions of force at high and low levels of MRLC phosphorylation.

We do not believe it is only a coincidence that the model was best fit when $C = 7$; i.e., when seven myosin binding sites were activated by binding of a cross bridge head. This finding suggests that the mediator of cooperative activation may be tropomyosin, given that skeletal muscle tropomyosin spans seven actins (4, 12). Clearly, this hypothesis needs to be tested. Our model suggests that a regulatory system beyond Ser19-MRLC phosphorylation may not be needed.

ACKNOWLEDGMENTS

We acknowledge that the data in Fig. 3 confirm earlier unpublished work by Dr. Seong-Chun Kwon in this laboratory. We did not incorporate Dr. Kwon’s data given methodological changes in the measurement of MRLC phosphorylation. We note that Dr. Kwon found a full sigmoidal dependence of force on MRLC phosphorylation when tissues were incubated at 22°C, much like the swine carotid at 37°C.

We thank Dr. John S. Walker and Bruce Gaylinn for providing insight and advice. We thank Gwaltney of Smithfield (Smithfield, VA) for contributing swine carotid arteries and Dr. James Stull for the gift of MRLC antibodies. Robin Woodson and Marcia Ripley provided technical assistance.

GRANTS

This research was supported by National Institutes of Health Grants HL-71191 and DK-56034 and by Mid-Atlantic American Heart Association Grants 0151586U and 0051587U.

REFERENCES