An expanded latch-bridge model of protein kinase C-mediated smooth muscle contraction

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Hai, Chi-Ming, and Hak Rim Kim. An expanded latch-bridge model of protein kinase C-mediated smooth muscle contraction. J Appl Physiol 98: 1356–1365, 2005. First published November 19, 2004; doi:10.1152/japplphysiol.00834.2004.—A thin-filament-regulated latch-bridge model of smooth muscle contraction is proposed to integrate thin-filament-based inhibition of actomyosin ATPase activity with myosin phosphorylation in the regulation of smooth muscle mechanics. The model included two latch-bridge cycles, one of which was identical to the four-state model as proposed by Hai and Murphy (Am J Physiol Cell Physiol 255: C86–C94, 1988), whereas the ultraslow cross-bridge cycle has lower cross-bridge cycling rates. The model-fitted phorbol ester induced slow contractions with constant myosin phosphorylation and predicted steeper dependence of force on myosin phosphorylation in phorbol ester-stimulated smooth muscle.

The inclusion of MLCP inhibition in the four-state model also accounted for the increase in intracellular 
Ca2+
 concentration ([Ca2+]i). In contrast, changes in myosin phosphorylation follow the changes in cross-bridge cycling rate, as measured by unloaded shortening velocity during smooth muscle contraction (4, 5). Therefore, steady-state force is maintained at relatively low levels of myosin light chain phosphorylation and cross-bridge cycling rates, which has been termed the “latch state” (5, 11). Hai and Murphy (9, 10) proposed a four-state cross-bridge model to explain the latch state in smooth muscle (Fig. 1A). The four-state model postulates the existence of attached, unphosphorylated latch bridges (AM) that are generated by dephosphorylation of attached phosphorylated cross bridges (AMP). The four-state model was able to predict the myosin phosphorylation transient during monotonic force development (9), linear dependence of cross-bridge cycling rate on myosin phosphorylation (10), and quasi-hyperbolic dependence of steady-state force on myosin phosphorylation (28). Using measurements of intracellular 
Ca2+
 concentration ([Ca2+]i), Rembold and Murphy (30) showed that the four-state model could quantitatively predict the force oscillations in response to intracellular [Ca2+]i oscillations. By adding length dynamics, Yu et al. (43) showed that the four-state model could explain both isometric and nonisometric responses of vascular smooth muscle, airway smooth muscle, molluscan catch muscle, and Aplysia muscle. By including twitch-dependent regulation of latch-bridge detachment, Butler et al. (3) showed that the four-state model could quantitatively explain the rate of catch muscle contraction by twitch phosphorylation. By incorporating Huxley’s (15) model of strain-dependent cross-bridge cycling rates, Miyailovitch et al. (23) showed that the four-state model could predict force fluctuation-induced lengthening of airway smooth muscle. These results show that the four-state model is robust in explaining smooth muscle mechanics.

However, the four-state model is incomplete because it does not include thin-filament-based regulatory mechanisms such as calponin and caldesmon (8, 24, 38, 41), and it cannot explain phorbol ester-induced slow contractions when myosin phosphorylation has already reached steady state (1, 13, 31, 33, 35, 37). Calponin and caldesmon are actin-binding proteins that inhibit smooth muscle actomyosin ATPase activity in vitro. Phorbol esters are activators of PKC and have been shown to induce slow smooth muscle contractions with little or no increase in intracellular [Ca2+]i and myosin phosphorylation. PKC-mediated inhibition of myosin light chain phosphatase (MLCP) activity could explain phorbol ester-induced contractions with small changes in intracellular [Ca2+]i (2, 21, 22, 29, 36, 42). However, this mechanism cannot explain phorbol ester-induced contractions at constant myosin phosphorylation. The inclusion of MLCP inhibition in the four-state model also

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could not predict force development at constant myosin phosphorylation, because the model would predict the loss of latch bridges as a result of MLCP inhibition (12).

Sato et al. (31) have performed quick release experiments on KCl- and phorbol dibutyrate (PDBu)-stimulated smooth muscle strips. They observed similar force recoveries after shortening in KCl- and PDBu-stimulated muscle strips. Therefore, Sato et al. (31) concluded that PDBu-induced contractions were mediated by cycling cross bridges. The phorbol ester-induced slow contraction data suggest the appearance of a population of ultraslow cycling cross bridges at constant myosin phosphorylation. In the four-state model, the number of latch bridges is determined by the relative rates of latch-bridge detachment, latch-bridge phosphorylation, and dephosphorylation of attached cross bridges. Therefore, slowing of cross-bridge cycling rates, including latch-bridge detachment, could potentially lead to force development at constant myosin phosphorylation. Accordingly, we added an ultraslow latch-bridge cycle to the four-state model to create a thin-filament-regulated latch-bridge model, as shown in Fig. 1B. The regular and ultraslow latch-bridge cycles both have the four-state structure but different cross-bridge cycling rates. In this study, we tested the ability of the thin-filament-regulated latch-bridge model to fit the data of phorbol ester-induced slow force development at constant myosin phosphorylation. In addition, we performed model simulations to test the model’s predictions of the steady-state dependencies of force and apparent cycling rate constants on myosin phosphorylation.

MATERIALS AND METHODS

Mathematical Modeling

The system of differential equations was solved using WinSAAM, the Windows version of SAAM (Simulation, Analysis, and Modeling), run on a Pentium III-based microcomputer, as described by Wastney et al. (39). The earlier version of this software, SAAM, has been used previously in our laboratory’s compartmental analysis of Ca2+ kinetics in vascular smooth muscle (27). Our laboratory’s published data on PDBu-induced contraction (13) were used for modeling in this study because the cross-bridge cycling rate constants for bovine tracheal smooth muscle were already available (9). Because the primary goal in this study was model development, a model fit was considered acceptable when the model fits the mean data points within standard error bars. Tissues from slaughterhouse for normal food processing are exempted from institutional review.

Tissue Preparation

Bovine tracheae were collected from a local slaughterhouse and transported to the laboratory in cold (4°C) physiological salt solution (PSS) of the following composition (in mM): 140.1 NaCl, 4.7 KCl, 1.2 Na2HPO4, 2.0 MOPS (pH 7.4), 0.02 Na2EDTA, 1.2 MgSO4, 1.6 CaCl2, and 5.6 d-glucose. The smooth muscle layer, together with the adventitial and mucosal layers, were excised from the trachea by making longitudinal cuts along their attachments to the cartilage. The adventitial and mucosal layers were then carefully dissected away in cold PSS using microdissecting scissors and fine forceps under a dissecting microscope. Smooth muscle strips were prepared by making cuts along the direction of smooth muscle bundles, corresponding to the circumferential direction, in vivo.

Isometric Contractions

One end of each muscle strip was clamped to a stainless steel clip connected to a force transducer (Grass FT.03), and the other end was clamped to a stainless steel clip connected to a length manipulator (Narishige). Muscle strips were then equilibrated for 2 h in PSS (37°C, pH 7.4) bubbled with air and adjusted to optimal length for maximal active force development, as described previously (20). Muscle strips were then allowed to relax in PSS for 1 h before stimulation by 1 μM PDBu for 30 min.

Tissue Homogenization, Fractionation, SDS-PAGE, and Western Blotting

After 30 min of stimulation by carbachol, muscle strips were quickly homogenized in a cold (4°C) extraction buffer of the following composition: 20 mM Tris·HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 6 mM mercaptoethanol, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 0.1 mM Na3VO4, 1 mM PMSF, and 50 mM NaF. Tissue homogenates were centrifuged at 100,000 g for 1 h to separate the cytoskeletal-membrane and cytoplasmic fractions. The pellet represents the cytoskeletal-membrane fraction, whereas the supernatant represents the cytosolic fraction, as described previously (20). After ultracentrifugation, pellets were homogenized in a SDS buffer (1% SDS, 10% glycerol, and 20 mM dithiothreitol) at 20 mg/ml. The supernatant volume was measured, and an equal volume of 2× SDS buffer was added. The pellet and supernatant samples were stored at −80°C.

Proteins were separated by SDS-PAGE using a separating gel of 7.5% acrylamide. Pellet and supernatant samples from the same tissue were loaded next to each other to minimize variations during Western blotting. Equal volumes of pellet and supernatant samples were loaded onto the same gel. After SDS-PAGE, proteins were transferred elec-
trophoretically to a nitrocellulose membrane (Transblot, Bio-Rad). The nitrocellulose membranes were blocked with 5% nonfat dry milk in Tris-buffered saline. After blocking, nitrocellulose membranes were incubated with primary antibodies against the conventional PKC isoforms \(\alpha\), \(\beta\), and \(\beta II\), and novel PKC isoforms \(\delta\) and \(\epsilon\) at 4°C with gentle shaking overnight. The conventional PKC isoforms \(\alpha\), \(\beta\), and \(\beta II\), and novel PKC isoforms \(\delta\) and \(\epsilon\) were selected for this study because PDBu, an analog of diacylglycerol, is expected to activate these two classes of PKC isoforms (14). Furthermore, bovine tracheal smooth muscle has been found to express predominantly PKC-\(\alpha\), -\(\beta I\), and -\(\beta II\), with very low expression levels of PKC-\(\delta\) and -\(\epsilon\) (40). Next, the nitrocellulose membranes were washed with Tris-buffered saline and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Primary antibodies against PKC isoforms were purchased from Santa Cruz. Secondary antibodies were purchased from Sigma.

Protein bands were detected by using enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ) and XAR Autoradiography film (Kodak, Rochester, NY). Film images were captured and analyzed by the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290) and Kodak 1D image analysis software. Because the loading volumes for pellet and supernatant samples were the same for each gel, we calculated the pellet-to-supernatant ratio (P/S) for each protein from pellet band density \(\times\) pellet sample volume/supernatant band density \(\times\) supernatant sample volume. P/S are presented as means \(\pm\) SE; \(n\) represents the number of animals. Student’s \(t\) test was used for the comparison of two means (\(P < 0.05\) was considered significant).

RESULTS

The Thin-Filament-Regulated Latch-Bridge Model

Figure 1A shows the four-state model that was proposed by Hai and Murphy (9). A major assumption of this model was the postulated existence of latch bridges (AM) that were generated by dephosphorylation of attached, phosphorylated cross bridges (AMP). Figure 1B shows the thin-filament-regulated latch-bridge model that includes two latch-bridge cycles, both having the four-state structure but different cycling rates. The regular latch-bridge cycle, consisting of M, Mp, AM, and AMP, and the cycling rate constants \(K_3\), \(K_4\), and \(K_5\) were identical to the four-state model proposed by Hai and Murphy (Fig. 1A). The ultralow latch-bridge cycle, consisting of \(M'\), \(M_{p'}\), \(A'M'\), and \(A'M_{p'}\), and the cycling rate constants \(K_{30}\), \(K_{40}\), and \(K_{70}\) also have a four-state structure but slower cross-bridge cycling rates than the regular latch-bridge cycle. The specific assumptions and initial conditions for the model are as follows.

**Assumption 1.** Myosin light chain kinase (MLCK) and MLCP activities are independent of cross-bridge states. This assumption was also included in the formulation of the four-state model by Hai and Murphy (9). This assumption implies that a single rate constant for MLCK activity \(K_1\) governs the phosphorylation of all unphosphorylated cross-bridge species (M, AM, \(M'\), and \(A'M'\)). Similarly, a single rate constant for MLCP activity \(K_2\) governs the dephosphorylation of all phosphorylated cross-bridge species (Mp, AMP, \(M_{p'}\), and \(A'M_{p'}\)).

**Assumption 2.** Attenuation of cross-bridge cycling rates in the ultralow latch-bridge cycle is independent of cross-bridge states. This assumption was made to avoid bias in selecting specific rate constants for attenuation. This assumption implies proportional attenuation of all rate constants for cross-bridge cycling in the ultralow latch-bridge cycle relative to the regular latch-bridge cycle. This assumption is described mathematically as follows.

\[
K_{30}/K_3 = K_{40}/K_4 = K_{70}/K_7 = a \text{ where } a < 1
\]

**Assumption 3.** A pair of transition rate constants \(K_9\) and \(K_{10}\) governs the exchange between the regular and latch-bridge cycles. We took the minimalist approach to assume a single point of exchange between \(A + M\) and \(A' + M'\) in the thin-filament-regulated latch-bridge model.

**Initial conditions.** We took the minimalist approach by assuming the following initial conditions before activation by PDBu.

\[
[M] = 1;
\[
[M] = [AM] = [M'] = [M'] = [AM'] = [M'] = 0
\]

The following system of differential equations describes the thin-filament-regulated latch-bridge model, as shown in Fig. 1B.

\[
d[M]/dt = K_3[M] + K_{10}[M] - (K_3 + K_4)[M]
\]
\[
d[AM]/dt = K_3[AM] + K_{10}[AM] - (K_4 + K_5)[AM]
\]
\[
d[Mp]/dt = K_3[Mp] + K_{10}[AM] - (K_5 + K_6)[AM]
\]
\[
d[AMp]/dt = K_3[AMp] + K_{10}[AM] - (K_7 + K_8)[AMp]
\]

\[
K_{30} \text{ and } K_{40} \text{ fit the time course of myosin phosphorylation (Fig. 2B) during PDBu-induced contractions of bovine tracheal smooth muscle, as published by Hai and Szeto (13). Step functions of MLCK activity were used to model the PDBu data because they required the minimum number of parameters. Basal myosin phosphorylation was not fitted because of the uncertainty on what mechanisms and artifacts contributed to basal myosin phosphorylation in the experimental data. Figure 2C shows the prediction of force by the four-state model using the same cross-bridge cycling rate constants \(K_3, K_4, K_5\), and \(K_7\), as published by Hai and Murphy (9) for bovine tracheal smooth muscle. Model prediction of force was calculated from the sum of AMP, AM, and basal force. As shown in Fig. 2C, the four-state model predicted the force data reasonably well up to 600 s of PDBu-induced contractions. The agreement between the model and data was remarkable because the rate constants for cross-bridge cycling \(K_3, K_4, K_5, K_7\) were constrained to be the same as those published by Hai and Murphy (9). However, the four-state model failed to predict force development after 600 s when myosin phosphorylation had already reached steady state. The failure of the four-state model to fit the later time points was to be expected because the rate constants for cross-bridge cycling, \(K_3, K_4, K_5, K_7\) were resolved previously from the fast contractions of...
electrically activated smooth muscle (9, 19, 34). Although Fig. 2C shows only one data point at 1,800 s, PDBu-induced slow-force development was continuous between 600 and 1,800 s (18, 33). Therefore, an important issue in modeling the PDBu data was whether a model could predict force development after myosin phosphorylation had already reached steady state.

Figure 3 shows fitting of the PDBu data by the thin-filament-regulated latch-bridge model. The same step functions of MLCK activity, as shown previously in Fig. 2A, were used to fit the myosin phosphorylation data. Changing the model did not change the fit to the myosin phosphorylation data (Fig. 3B) because MLCK (K₁) and MLCP (K₂) activities were modeled as independent of cross-bridge states. Figure 3C shows that the thin-filament-regulated latch-bridge model was able to fit

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**Fig. 2.** Fitting of experimental data of Hai and Szeto (13) (● and dashed lines) by four-state model of Hai and Murphy (9) (solid lines). A: time course of K₁ (MLCK activity) that generated the model fit to the myosin phosphorylation (solid line; B). K₁ (MLCK activity) was 4.5 × 10⁻³ s⁻¹ between 0 and 60 s, 5 × 10⁻⁴ s⁻¹ between 61 and 180 s, and 2.1 × 10⁻³ s⁻¹ between 181 and 1,800 s. K₂ (MLCP activity) was 8 × 10⁻³ s⁻¹. C: model prediction of force (solid line) using the same cross-bridge cycling rate constants (K₃, K₄, and K₇) as published by Hai and Murphy (9) for bovine tracheal smooth muscle. K₃ was 0.44 s⁻¹, K₄ was 0.11 s⁻¹, and K₇ was 0.005 s⁻¹. PDBu, phorbol dibutyrate; Fₒ, maximum isometric force.

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**Fig. 3.** Fitting of experimental data by Hai and Szeto (13) (● and dashed lines) by the thin-filament-regulated latch-bridge model (solid lines). A: time course of K₁ (MLCK activity) that generated the model fit to the myosin phosphorylation data (solid line; B). K₁ (MLCK activity) was 4.5 × 10⁻³ s⁻¹ between 0 and 60 s, 5 × 10⁻⁴ s⁻¹ between 61 and 180 s, and 2.1 × 10⁻³ s⁻¹ between 181 and 1,800 s. K₂ (MLCP activity) was 8 × 10⁻³ s⁻¹. C: model prediction of force by the thin-filament-regulated latch-bridge model (solid line) that fitted the experimental data (symbols). The cross-bridge cycling rate constants (K₃, K₄, and K₇) for the ultraslow latch-bridge cycle were proportionally slower than the corresponding rate constants (K₃, K₄, and K₇) in the regular latch-bridge cycle. K₃, K₄, and K₇ were the same as those published by Hai and Murphy (9) for bovine tracheal smooth muscle. K₃ was 0.44 s⁻¹, K₄ was 0.11 s⁻¹, and K₇ was 0.005 s⁻¹. PDBu, phorbol dibutyrate; Fₒ, maximum isometric force.
PDBu-induced continuous force development after myosin phosphorylation had reached steady state. As shown in Fig. 4, analysis of the cross-bridge states indicated that accumulation of the latch bridges A’M’ in the ultraslow latch-bridge cycle was primarily responsible for the slow-force development in PDBu-induced contractions.

**Sensitivity Analysis**

In the thin-filament-regulated latch-bridge model, proportional slowing of cross-bridge cycling rates was represented by the slowing factor ("a"), which equals the ratios of cross-bridge cycling rate constants between the ultraslow and regular cross-bridge cycles. As shown in Fig. 3C, the PDBu-induced contraction data were fitted with a slowing factor $a$ of 0.027, implying that the rate constants for cross-bridge cycling in the ultraslow cross-bridge cycle were 2.7% of the corresponding rate constants in the regular cross-bridge cycle. Fig. 5A shows the sensitivity analysis of the predicted force to changes in the slowing factor $a$. The solid line, labeled "a = 0.027" in Fig. 5A, represents the model’s fit to the PDBu-induced force development. The dashed line, labeled "a = 1" in Fig. 5A, represents the special case of identical cross-bridge cycling rates in the two latch-bridge cycles. In this special case, the thin-filament-regulated latch-bridge cycle becomes the same as the four-state model. Therefore, the thin-filament-regulated latch-bridge model represents a general model that includes the four-state model as a special case when the two latch-bridge cycles have identical cross-bridge cycling rates. As shown in Fig. 5A, as the value of the slowing factor $a$ decreases, the model predicts force development at constant myosin phosphorylation.

As shown in Fig. 1B, the rate constant $K_9$ governs the transition from the regular latch-bridge cycle to the ultraslow latch-bridge cycle. Figure 5B shows the sensitivity analysis of the predicted force to changes in the transition rate constant $K_9$. The solid line, labeled "3E-3" in Fig. 5B, represents the model’s fit to PDBu-induced force development. As shown in the dashed lines in Fig. 5B, other values of $K_9$ failed to fit the rate of force development in PDBu-induced contractions.

![Diagram showing sensitivity analysis](http://jap.physiology.org/)

**Steady-State Analysis**

Using the set of rate constants that fitted the PDBu data (Fig. 3), steady-state solutions of the cross-bridge states were computed for different values of myosin phosphorylation. Figure 6A shows the model’s prediction of the dependence of steady-state force on myosin phosphorylation, and the steady-state distribution of the four attached cross-bridge states as functions of myosin light chain phosphorylation. As shown in Fig. 6A, the model predicted a quasi-hyperbolic dependence of force on myosin phosphorylation, such that steady-state force reached 90% of maximum force at ~30% myosin phosphorylation (Fig. 6A, solid line). A’M’ and A’M’ in the ultraslow latch-bridge cycle were the major attached cross bridges that contributed to force development (Fig. 6A). A’M in the regular latch-bridge cycle also increased with myosin phosphorylation, but [A’Mp] was only one-third or less of [A’Mp] at a given state.

![Diagram showing steady-state analysis](http://jap.physiology.org/)
level of myosin phosphorylation. [A'Mp'] was approximately threefold of [AMP] at a given level of myosin phosphorylation due to the 3:1 ratio of \(K_9/K_{10}\). The accumulation of A'M at low levels of myosin phosphorylation was due to the slow rate constant of latch-bridge detachment for A'M (\(K_{70} = 0.00014 \) s\(^{-1}\)) relative to MLCK activity (\(K_1\)) and MLCP activity (\(K_2\)). In the steady-state analysis, \(K_1\) ranged from 0.001 s\(^{-1}\) at 11% myosin phosphorylation to 0.1 s\(^{-1}\) at 92% myosin phosphorylation, whereas \(K_2\) was 0.008 s\(^{-1}\). As shown in the thin filament-regulated latch-bridge model (Fig. 1B), when the latch-bridge detachment rate constant \(K_{70}\) becomes negligible relative to \(K_1\), [A'M'] is determined primarily by the exchanges with A'Mp', via MLCK \((K_1)\) and phosphatase \((K_2)\) activities. Therefore, except at a very low value of MLCK activity \((K_1)\), the [A'M']/[A'Mp'] could be approximated by the MLCP-to-MLCK ratio. As a result, [A'M'] was higher than [A'Mp'] when myosin phosphorylation was <50%. Conversely, [A'M'] was lower than [A'Mp'] when myosin phosphorylation was >50%.

Figure 6B shows the effect of changing the transition rate constant \(K_9\) on the steady-state force-myosin phosphorylation relation. As shown in Fig. 6B, a 10-fold decrease in \(K_9\) from \(3 \times 10^{-3} \) to \(3 \times 10^{-2}\) s\(^{-1}\) substantially decreased the slope of the steady-state phosphorylation-force relation. In contrast, a 10-fold increase in \(K_9\) from \(3 \times 10^{-3} \) to \(3 \times 10^{-2}\) s\(^{-1}\) had a relatively small effect on the slope of the steady-state phosphorylation-force relation. The differential sensitivities of the force-myosin phosphorylation relation to the increase and decrease in \(K_9\) can be explained in terms of the fraction of total cross-bridge population that is engaged in the ultraslow cross-bridge cycle. At the value of \(K_9\) that fitted the PDBu data (Fig. 3), the \(K_9\)-to-\(K_{10}\) ratio was 3:1, implying that ~75% of the cross-bridge population was engaged in the ultraslow latch-bridge cycle during PDBu-induced contractions. A 10-fold decrease in \(K_9\) would decrease the \(K_9\)-to-\(K_{10}\) ratio to 0.3:1, decreasing the cross-bridge population engaged in the ultraslow latch-bridge cycle to 23%. This represents a relatively large loss (69%) of the original cross-bridge population and results in a relatively large decrease in the slope of the force-phosphorylation relation. In contrast, a 10-fold increase in \(K_9\) from \(3 \times 10^{-3} \) to \(3 \times 10^{-2}\) s\(^{-1}\) would increase the \(K_9\)-to-\(K_{10}\) ratio to 30:1, increasing the cross-bridge population engaged in the ultraslow latch-bridge cycle to 97%. This represents a relatively small gain (29%) of the original cross-bridge population and results in a relatively small increase in the slope of the force-myosin phosphorylation relation.

Model Simulations

Modeling of PDBu data predicts that shifting cross bridges from the regular latch-cycle to the ultraslow latchbridge cycle will lead to force development at constant myosin phosphorylation. To test this model prediction directly, we performed the following model simulations. First, a step increase in MLCK activity \((K_1)\) was simulated to induce a relatively fast increase in myosin phosphorylation that reached steady state within 1,000 s (Fig. 7A). All other rate constants were constrained to be the same as those that fitted the PDBu data (Fig. 3). Figure 7B shows the three model simulations of changing \(K_9\) and the predicted time courses of force development. It is important to note that changing \(K_9\) has no effect on myosin phosphorylation because the rate constants for MLCK and MLCP activities were modeled as independent of cross-bridge states. When \(K_9\) was held at \(3 \times 10^{-3}\) s\(^{-1}\) at all times between 0 and 7,000 s, the model predicted monotonic development of force to ~70% of total cross bridges (Fig. 7B, “\(K_9 = 3E-3\)”). When \(K_9\) was held at \(3 \times 10^{-4}\) s\(^{-1}\) at all times between 0 and 7,000 s, the model predicted monotonic development of force to ~50% of total cross bridges (Fig. 7B, “\(K_9 = 3E-4\)”). When \(K_9\) was held at \(3 \times 10^{-4}\) s\(^{-1}\) from 0 to 1,880 s and then increased to \(3 \times 10^{-3}\) s\(^{-1}\) at 1,881 s, the model predicted the following. As shown in Fig. 7B (solid line), the predicted force first followed the curve for \(K_9 = 3 \times 10^{-4}\) s\(^{-1}\) up to 1,880 s, then decreased slightly, and finally increased to the level for \(K_9 = 3 \times 10^{-3}\) s\(^{-1}\) while myosin phosphorylation remained constant at all times.
bridges from regular latch-bridge cycle to ultraslow latch-bridge cycle. As shown in Fig. 8A, an increase in $K_9$ led to the even exchange of the phosphorylated cross bridges $M_p$ and $AMp$ in the regular latch-bridge cycle, with the phosphorylated cross bridges $M'_p$ and $A'M'p$ in the ultraslow latch-bridge cycle, without changing the level of myosin phosphorylation. This exchange was even because the rate constants for MLCK and MLCP activities were modeled as independent of cross-bridge states. As shown in Fig. 8B, an increase in $K_9$ led to the exchange of the attached cross bridges $A'M'p$ and $A'M$ in the ultraslow latch-bridge cycle, with the attached cross bridges $AMp$ and $AM$ in the regular latch-bridge cycle. However, the exchange of attached cross bridges between the two latch-bridge cycles was uneven, resulting in the net gain of attached cross bridges in the ultraslow latch-bridge cycle and force development.

**Apparent Cross-Bridge Cycling Rate Constants**

Apparent attachment and detachment rate constants were calculated from the steady-state solutions (Fig. 9). As described previously by Hai and Murphy (10), the apparent cross-bridge attachment rate constant ($F$) was defined as follows: (total flux of cross-bridge attachment)/(total detached cross bridges). In a steady state, the flux along a given pathway equals mass $\times$ rate constant (39). Accordingly, the following equation calculates the apparent attachment rate constant.

$$F = (K_a[M_p] + K_3[M'_p])/(M[M] + [M'] + [M'_p])$$

The apparent detachment rate constant ($G$) was defined as follows: (total flux of cross-bridge detachment)/(total attached cross bridges). The following equation calculates the apparent detachment rate constant.


Figure 9 shows the dependencies of apparent attachment and detachment rate constants on myosin phosphorylation at different values of $K_9$. At a given value of $K_9$, the increase in apparent cycling rate constants with myosin phosphorylation
reflected the conversion of latch bridges to the cycling phosphorylated cross bridges within each of the two latch-bridge cycles. The $K_9$-dependent changing of the dependence of apparent cycling rate constants on myosin phosphorylation (Fig. 9) reflects the shifting of cross bridges from the regular latch-bridge cycle to the ultraslow latch-bridge cycle.

**PDBu-Induced Translocation of PKC Isoforms**

Figure 10 shows the Western blots of PKC-$\alpha$-, $\beta$, and $\beta$II in the pellet and supernatant fractions of unstimulated (PSS) and 1 $\mu$M PDBu-stimulated (PDBu) tissues. We observed extremely low expression levels of PKC-$\delta$ and $\varepsilon$ that were indistinguishable from background signals. This observation is consistent with the findings of Webb et al. (40). As shown in Fig. 10, in unstimulated tissues (PSS), band densities for PKC-$\alpha$, $\beta$, and $\beta$II were stronger in the supernatant than the pellet, whereas the reverse is true for PDBu-stimulated tissues (PDBu). As shown in Fig. 11, PDBu stimulation significantly increased the P/S of PKC-$\alpha$ and $\beta$ by 3.0- and 5.9-fold, respectively. However, the increase in the P/S of PKC-$\beta$II was statistically insignificant.

**DISCUSSION**

**Four-State Model vs. Thin-Filament-Regulated Latch-Bridge Model**

In this study, a new model of smooth muscle contraction (Fig. 1B) has been developed to address some experimental data that could not be explained by the four-state model (Fig. 1A). Thin-filament-based regulation of actomyosin ATPase activity was absent in the four-state model because the four-state model assumed that myosin light chain phosphorylation was the only regulatory mechanism (9, 10). Phorbol ester-induced force development at constant myosin phosphorylation could not be explained by the four-state model because the rate constants for cross-bridge cycling were derived from fast contractions of electrically stimulated smooth muscle (9, 19). The proposed thin-filament-regulated latch-bridge model included two latch-bridge cycles, one of which was identical to the four-state model, whereas the ultraslow cross-bridge cycle had lower cross-bridge cycling rates. To avoid bias in selecting specific rate constants for attenuation, we assumed propor-

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**Fig. 9.** Model predictions of variable dependencies of apparent attachment rate constant ($F$) (A) and apparent detachment rate constant ($G$) on myosin phosphorylation as a function of $K_9$ (B). Apparent attachment and detachment rate constants were calculated using the rate constants and steady-state solutions shown in Fig. 6 (see text).

**Fig. 10.** Western blots of PKC-$\alpha$, $\beta$, and $\beta$II in the pellet (P) and supernatant (S) fractions of unstimulated [physiological salt solution (PSS)] and 1 $\mu$M PDBu-stimulated bovine tracheal smooth muscle.

**Fig. 11.** Pellet-to-supernatant ratios (P/S) of PKC-$\alpha$, $\beta$, and $\beta$II in unstimulated and 1 $\mu$M PDBu-stimulated bovine tracheal smooth muscle. Identical volumes of pellet (P) and supernatant (S) samples were loaded into adjacent lanes of the same gel to minimize variations during Western blotting. Total pellet and supernatant volumes from a given tissue were similar. The P/S were calculated from pellet band density/supernatant band density. Data are presented as means $\pm$ SE (n = 3). P/S represents the partition of a given protein between the cytoskeletal/membrane complex and cytoplasm. *Significant difference between P/S in unstimulated and PDBu-stimulated tissues ($P < 0.05$).
tional slowing of all cross-bridge cycling rate constants in the ultraslow latch-bridge cycle. The thin-filament-regulated latch-bridge model was able to fit phorbol ester-induced ultraslow contractions at constant myosin phosphorylation by shifting cross bridges from the regular latch-bridge cycle to the ultraslow cross-bridge cycle. This modeling result was counterintuitive because proportional slowing of cross-bridge attachment and detachment rates was not expected to change the number of attached cross bridges in a two-state cross-bridge model. Modeling the phorbol ester data indicated that the rate constants for cross-bridge cycling in the ultraslow cross-bridge cycle were only 2.7% of those in the regular cross-bridge cycle. Therefore, adding the ultraslow latch-bridge cycle to the four-state model would increase the dynamic range of cross-bridge cycling rate by 37-fold without losing the capacity to generate force. Functionally, having the regular and ultraslow cross-bridge cycles would allow a smooth muscle cell to have the flexibility of either maintaining force at extremely low-energy cost or changing its contractile state rapidly, if necessary.

Implications for PKC-Mediated Smooth Muscle Contraction

A large amount of data derived from phorbol ester experiments suggest the existence of mechanisms that can generate ultraslow contractions without changing myosin phosphorylation in smooth muscle (7, 13, 18, 29, 31, 33). PKC-mediated phosphorylation of CPI-17 has been postulated as a mechanism of PKC-mediated inhibition of MLCP (17). This mechanism can potentially explain the initial increase in myosin light chain phosphorylation with a modest increase in intracellular [Ca$^{2+}$] in PDBu-induced contractions. However, this mechanism cannot explain the continued force development after myosin phosphorylation has already reached steady state. Fulginiti et al. (7) found that the stress-stiffness relationship was not significantly different between K+-depolarized and phorbol ester-activated smooth muscle, suggesting that cross bridges are the basic force-bearing element in phorbol ester-activated smooth muscle. Therefore, a challenge to explaining the phorbol ester data is to develop a cross-bridge model that can predict phosphorylation-independent smooth muscle contractions.

In this study, we modeled the PDBu data by adding an ultraslow cross-bridge cycle to the four-state model, with the implicit assumption that PKC activation leads to the inhibition of actomyosin ATPase activity. This assumption is consistent with the findings of Nishikawa et al. (25) and Ikebe et al. (16) that PKC phosphorylates smooth muscle myosin, leading to the inhibition of actin-activated myosin ATPase activity. Several studies have confirmed that the PKC-specific sites on smooth muscle myosin were indeed phosphorylated in PDBu-activated arterial smooth muscle (1, 32, 35). In this study, we studied the translocation of conventional and novel PKC isoforms in PDBu-induced contractions. The observed PDBu-induced significant increases in the P/S of PKC-α and -β suggest that these two conventional PKC isoforms are involved in PDBu-induced contractions (Figs. 10 and 11). The relatively small and insignificant change in the PKC-βII P/S could not explain the 5.9-fold increase in the P/S of PKC-β in response to PDBu stimulation, suggesting that other PKC-β isoforms, possibly PKC-βI, are involved in PDBu-induced contractions. These results are consistent with the hypothesis that PDBu-induced airway smooth muscle contraction is mediated by the activation of conventional PKC isoforms.

Therefore, it is conceivable that PDBu inhibits actomyosin ATPase activity in intact smooth muscle. Although myosin is the postulated target of PKC in phorbol ester-induced contractions, the thin-filament-regulated latch-bridge model remains applicable because, mathematically, the model only assumes attenuation of actomyosin ATPase activity in the ultraslow latch-bridge cycle without specifying the target of regulation. As shown in this study, the thin-filament-regulated latch-bridge model fitted the PDBu-induced slow contractions at constant myosin phosphorylation. Furthermore, steady-state solutions of the model predicted steeper dependence of force on myosin phosphorylation in PDBu-stimulated smooth muscle, consistent with the findings by Bremerich et al. (2). These modeling results suggest that PKC-mediated inhibition of actomyosin ATPase activity may be an important mechanism of phorbol ester-induced smooth muscle contractions.

Implications for Thin-Filament-Based Regulation of Smooth Muscle Contraction

Calponin and caldesmon are thin-filament-based regulatory proteins that inhibit actomyosin ATPase activity in vitro. Calponin knockout mice studies indicated that calponin attenuated maximum shortening velocity independent of myosin phosphorylation in intact smooth muscle (38). On the contrary, Facemire et al. (6) did not observe a significant difference in shortening velocity between smooth muscles naturally expressing different amounts of calponin. Therefore, the physiological role of calponin in regulating smooth muscle mechanics remains controversial. The model described in this study provides a specific and mechanistic framework for integrating thin-filament-based inhibition of actomyosin ATPase activity with myosin light chain phosphorylation in the regulation of smooth muscle mechanics. The model postulates the existence of two latch-bridge cycles with different cross-bridge cycling rates. This postulate is compatible with the biochemical and thin-filament-based regulation. First, the model predicts biphasic kinetics of smooth muscle mechanics as illustrated by phorbol ester-activated contractions. Second, the model predicts variable steady-state dependencies of force and shortening velocity on myosin phosphorylation, depending on the fraction of total cross bridges engaged in the calponin-inhibited ultraslow latch-bridge cycle. These model predictions highlight the importance of considering biphasic kinetics, calponin phosphorylation, and myosin phosphorylation in studying the effect of calponin and caldesmon on smooth muscle mechanics. Finally, modeling results from this study suggest the novel concept that thin-filament-based regulatory proteins may function as tuners of actomyosin ATPase activity, thus allowing a smooth muscle cell to have two discrete cross-bridge cycles with substantially different cross-bridge cycling rates.
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REFERENCES


