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Alteration of cross-bridge kinetics by myosin light chain phosphorylation in rabbit skeletal muscle: Implications for regulation of actin–myosin interaction

(contraction/modulation of force)

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ABSTRACT **Myosin light chain phosphorylation in permeable skeletal muscle fibers increases isometric force and the rate of force production at submaximal levels of calcium activation; myosin light chain phosphorylation may underlie the increased rate and extent of force production associated with isometric twitch potentiation in intact fibers. To understand the mechanism by which myosin light chain phosphorylation manifests these effects, we have measured isometric force, isometric stiffness, rate of isometric force redevelopment after isotonic shortening, and isometric ATPase activity in permeabilized rabbit psoas muscle fibers. These measurements were made in the presence and absence of myosin light chain phosphorylation over a range of calcium concentrations that caused various levels of activation. The results were analyzed with a two-state cross-bridge cycle model as suggested by Brenner [Brenner, B. (1988) *Proc. Natl. Acad. Sci. USA* 85, 3265–3269]. The results indicate that myosin light chain phosphorylation exerts its effect on force generation and the isometric rate of force redevelopment in striated muscle through a single mechanism, namely, by increasing the rate constant describing the transition from non-force-generating cross-bridges to force-generating states (f_{app}). g_{app}, the reverse rate constant, is unaffected by phosphorylation as are the number of cycling cross-bridges. Since both calcium and myosin light chain phosphorylation increase f_{app}, the possibility is considered that modulation of f_{app} may represent a general mechanism for regulating force in actin–myosin systems.**

Ca^{2+}-dependent myosin light chain phosphorylation was first discovered in skeletal muscle in 1972 (1). Since that discovery, myosin light chain phosphorylation has been shown to be a primary mechanism for initiating contraction in smooth muscle and in some nonmuscle systems (2–4). There has been much confusion surrounding its role in skeletal muscle. Early work demonstrated that decreases in shortening velocity and oxygen consumption correlated with myosin light chain phosphorylation (5, 6). However, subsequent work in skinned and intact skeletal muscle fibers did not support a causal relationship (7–9). The only known physiological correlation to myosin light chain phosphorylation is an increase in the rate of force development and potentiation of isometric twitch tension in fast-twitch skeletal muscle fibers (10–13). Myosin light chain phosphorylation causes an increase in force production at low levels of calcium activation (14, 15) and also causes an increased rate of force development (16) over a wide range of activation levels in permeable skeletal muscle fibers. These observations support the proposal that the physiological correlation between myosin light chain phosphorylation to increased twitch force and rate of force development is causal. However, a thorough understanding of how myosin light chain phosphorylation modulates the actin–myosin interaction in fibers is needed to provide insights into the molecular properties of these interactions, in addition to providing direction to future biochemical and biophysical studies on the interactions of the regulatory light chain with myosin heavy chain.

We adopted the analytical framework suggested by Brenner (17) to interpret the mechanical and ATPase data obtained in this study. In this analysis the multiple states of the cross-bridge kinetic cycle are reduced to a two-state model with the apparent rate constants, f_{app} and g_{app}. The transition from non-force-generating state to the force-generating state is described by f_{app}, whereas g_{app} describes the transition from the force-generating state back to the non-force-generating state. Thus the steady-state fraction of cycling cross-bridges in the force-generating state is given by:

$$\alpha_{Fs} = f_{app}/(f_{app} + g_{app}). \quad [1]$$

Isometric force (F) and isometric stiffness (S) are then

$$F = n\bar{F}\alpha_{Fs} \quad [2]$$

and

$$S = n\bar{S}\alpha_{Fs}, \quad [3]$$

where n is the number of cycling cross-bridges per half sarcomere, $\bar{F}$ is the mean force per cross-bridge in the force-generating state, and $\bar{S}$ is the mean stiffness of a cross-bridge in the force-generating state. The isometric ATP hydrolysis rate (r_A) of a fiber is

$$r_A = nbg_{app}\alpha_{Fs}, \quad [4]$$

where b is the total number of half sarcomeres within the fiber segment. The rate constant describing force redevelopment after isotonic shortening is then given by:

$$K_{redev} = f_{app} + g_{app}. \quad [5]$$

This analytical framework is discussed in detail in ref. 17.

Although this analysis combines a number of specific molecular transitions in the cross-bridge kinetic cycle, it can prove useful in examining whether myosin phosphorylation affects the number of cycling cross-bridges or the composite steps into or out of the force-generating states. This framework is based essentially on the earlier cross-bridge model of Huxley (18) for force generation in skeletal muscle, which has provided much of our current understanding of the mechanism of muscle contraction. The major assumption made in this experimental approach is that the processes underlying

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force redevelopment after isotonic shortening reflect isometric rate constants. This assumption has been verified in experiments comparing the rate of isometric force redevelopment to the rate of force development in a rigor fiber after flash photolysis of ATP (19). The rate constant describing force redevelopment in both experimental paradigms was the same.

MATERIALS AND METHODS

Permeabilized Fiber Preparation. Single permeabilized psoas fibers from adult female New Zealand White rabbits (2–3 kg) were prepared as described (7). This procedure results in loss of endogenous calmodulin, myosin light chain kinase, and myosin light chain phosphatase activities. Permeabilized fiber segments were attached with cyanoacrylate glue between the output shaft of a force transducer (Cambridge Technology, Cambridge, MA, model 403) and the lever arm of a high-speed servo-controlled motor (Cambridge Technology, model 300S). The fibers were suspended in a 50-μl flow-through chamber.

Solutions. Single-fiber segments were alternately bathed in either a relaxing solution or an activating solution. The solutions differed only in their pCa ($-\log[Ca^{2+}]$) values; relaxing solutions had no added free calcium (pCa $\approx$ 8), and activating solutions contained free calcium concentrations of pCa 6.0, 5.8, 5.7, 5.6, 5.5, 5.4, 5.2, 5.0, 4.8, 4.5, and 4.3. All solutions other than those used for ATPase measurements contained 4 mM EGTA, 5 mM MgATP, 15 mM creatine phosphate, creatine kinase (200 units/ml), 1 mM inorganic phosphate, 3 mM free magnesium, 100 mM free potassium, 10 mM free sodium, >70 mM propionate, and sufficient imidazole (>30 mM) to bring the ionic strength to 0.2 M. The pH was buffered at 7.1 and the temperature of the solution was maintained at 15°C. The concentrations of all ionic species were calculated by solving the multiple equilibria with an iterative computer program using published association constants (20). For experiments involving ATPase measurements, creatine phosphate and creatine kinase were eliminated from solutions and replaced by phospho*enol*pyruvate (15 mM) and pyruvate kinase ($\approx$5000 units/ml). Additionally, lactic dehydrogenase (500 units/ml) and NADH (0.5 mM) were added to allow ATP utilization to be coupled with NADH conversion to NAD^+.

Experimental Apparatus and Protocol. The mechanical apparatus and procedures used for isometric and isotonic measurements as well as the stabilization of single-fiber striation pattern have been described (21). The mechanical apparatus has been modified to allow laser light diffraction for measurement and servo-control of sarcomere length. The procedures and equipment used for this purpose are as described (22, 23). Servo-control of sarcomere length is critical during measurements of isometric force redevelopment after isotonic shortening (22). The apparatus has also been modified to allow simultaneous ATPase measurements. This modification involves a coupled fluorometric assay and is implemented as described by Güth and Wojciechowski (24). In-phase isometric stiffness was measured utilizing 3-kHz sinusoidal perturbations (0.05% fiber length at a sarcomere length of 2.5 μm). The rate constant for force redevelopment after isotonic shortening (K_{redev}) was determined from the slope of the relationship between $\ln(\Delta f)$ versus the time after restretch. Δf is defined as the difference between isometric force (prior to shortening) and instantaneous force (after restretch) during redevelopment (22).

The experimental protocol involved activating the permeabilized psoas fiber segments at 15°C over the pCa range of 6.0–4.3 (the order of the activations was varied so that no systematic differences due to activation order would be introduced). Isometric force, isometric stiffness, isometric ATPase activity, and the isometric rate constant K_{redev} were measured at each calcium level. Myosin light chain phosphorylation was then initiated by the addition of 2 μM calmodulin and 0.15 μM myosin light chain kinase. After a 15-min preincubation in relaxing solution, the fibers were briefly activated at pCa 4.3 to allow myosin light chain phosphorylation to occur. At the end of each experiment myosin light chain phosphorylation was measured. Myosin light chain phosphorylation levels were >75% after treatment with myosin light chain kinase and were stable in the absence of calcium. Control values were <10%. These results are consistent with previous observations (7, 14, 15). After phosphorylation of myosin light chain, mechanical and ATPase measurements were repeated from pCa 6.0 to pCa 4.3. Significant differences in mechanical responses to myosin light chain phosphorylation were determined by a paired Student's t test for intrafiber comparisons before and after phosphorylation.

RESULTS

Myosin light chain phosphorylation increased isometric force and the rate constant for isometric force redevelopment (K_{redev}), as shown in Fig. 1. The effect of myosin light chain phosphorylation on isometric force was more pronounced at low levels of calcium activation—i.e., from pCa 5.7 to pCa 5.5. However, the effect on the rate constant for isometric force redevelopment is greatest at higher levels of calcium activation. These results are in agreement with previous reports (15, 16).

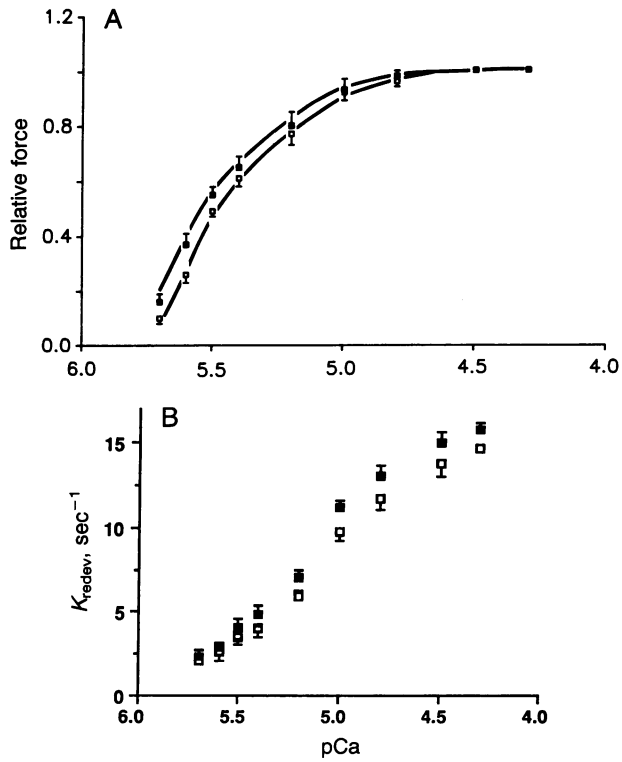

FIG. 1. Effect of myosin light chain phosphorylation on force production and the rate constant describing isometric force redevelopment at various levels of calcium activation. (*A*) Force values for 10 rabbit psoas fibers before (□) and after (■) phosphorylation. All values are expressed as a fraction of the force at pCa 4.3 prior to phosphorylation. (*B*) K_{redev} values for the same group of fibers before (□) and after (■) phosphorylation expressed as a fraction of the prephosphorylation K_{redev} value at pCa 4.3. Bars indicate SEM. Force was significantly increased with phosphorylation from pCa 5.8 to pCa 5.0, whereas K_{redev} was significantly increased from pCa 5.4 to pCa 4.3.

Fig. 2 summarizes the effect of myosin phosphorylation at various calcium activation levels on isometric stiffness and isometric rate of ATP utilization. As can be seen in Fig. 2*A*, the increases in fiber stiffness associated with phosphorylation are proportional to the increases in force. Thus, the same linear relationship was observed between force and stiffness for phosphorylated and nonphosphorylated cross-bridges. A similar linear relationship exists between force and ATPase activity (Fig. 2*B*). The results shown in Fig. 2 indicate that g_{app} is constant over all levels of calcium activation, whether or not myosin is phosphorylated. The reasoning that allows this conclusion is as follows. The fact that the ratio of force to stiffness is a constant implies that the ratio of the mean force per cross-bridge to the mean stiffness per cross-bridge ($\overline{F}/\overline{S}$) is a constant (Eq. **2** divided by Eq. **3**). Furthermore, the fact that the ratio between ATPase activity and force is a constant implies that the ratio of mean force per cross-bridge to g_{app} ($\overline{F}/g_{app}$) is a constant (Eq. **4** divided by Eq. **3**) (note that b is a constant). Thus, either (*i*) g_{app}, $\overline{F}$, and $\overline{S}$ all change exactly in proportion under all conditions of calcium activation and phosphorylation or (*ii*) all three values are constant over all conditions. Since the latter seems much more likely, we conclude that g_{app} is constant whether or not myosin light chains are phosphorylated and irrespective of the level of calcium activation. The constancy of g_{app} in turn implies that the effect of myosin phosphorylation on K_{redev} must be due to altering the value of f_{app}.

To calculate the magnitude of the effect of phosphorylation on f_{app}, an estimate of the value of g_{app} must be utilized. Estimates of g_{app} in rabbit psoas fibers at a variety of temperatures have been made (25). At 15°C the value of g_{app} was approximately 2 s^{-1}. Since Brenner's value (25) of K_{redev} at 15°C at maximal activation was the same as our measured value, we adopted his value of g_{app} for calculations. The calculated values of f_{app} are increased with myosin light chain phosphorylation over a large range of pCa values (Fig. 3*A*). The fraction of cycling cross-bridges in the force-generating states ($f_{app}/K_{redev} = \alpha_{Fs}$) increases as pCa decreases (Fig. 3*B*). These values of α_{Fs} can be used to calculate the values of the product $n\overline{F}$ (Eq. **2**). Fig. 4 shows the relationship of the

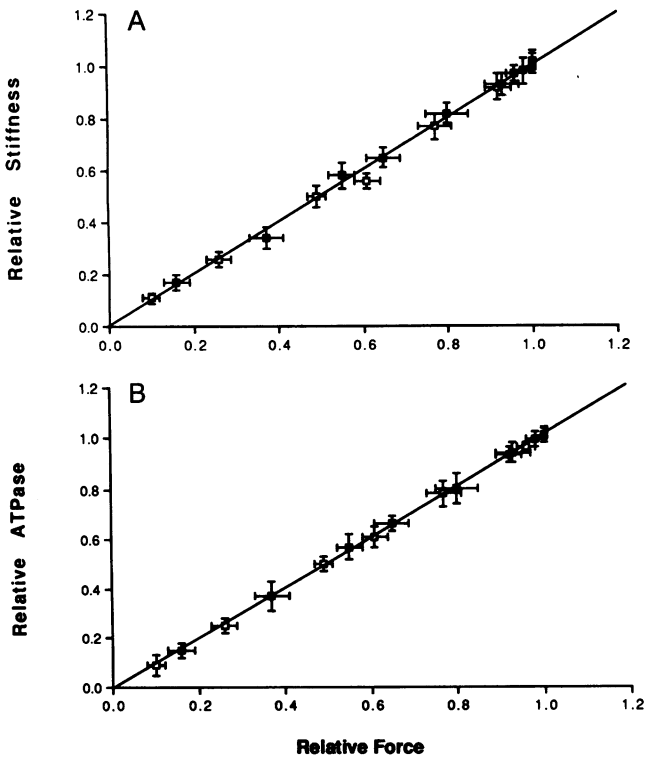

FIG. 2. Effect of myosin light chain phosphorylation on stiffness and ATPase activity. (*A*) Relationship between isometric stiffness and isometric force between pCa 5.7 and pCa 4.3 for 10 rabbit psoas fibers. Values before (□) and after (■) phosphorylation are represented as a fraction of the prephosphorylation value at pCa 4.3. (*B*) Relationship between isometric rate of ATP hydrolysis (ATPase) and isometric force (pCa 5.7–pCa 4.3). Values before (□) and after (■) phosphorylation are given as a fraction of the prephosphorylation value at pCa 4.3. Bars denote SEM.

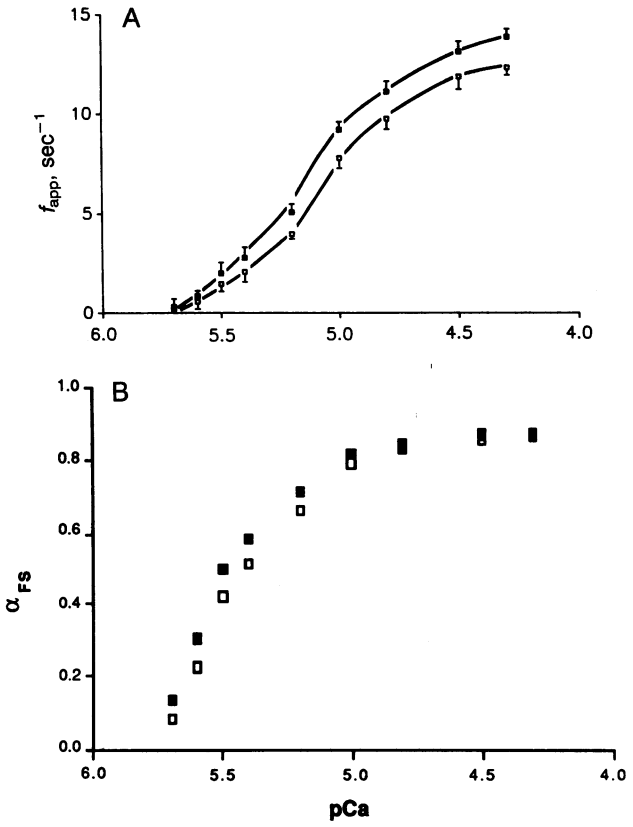

FIG. 3. Effect of myosin light chain phosphorylation on cross-bridge kinetic properties. (*A*) Calculated values of f_{app}. Values of f_{app} before (□) and after (■) phosphorylation are plotted as a function of pCa. (*B*) Fraction of cross-bridges in the force-producing states (α_{Fs}) plotted as a function of pCa. Values of α_{Fs} were calculated as $f_{app}/(f_{app} + g_{app})$, using the values of f_{app} in *A* and 2 s^{-1} for g_{app}, before (□) and after (■) phosphorylation.

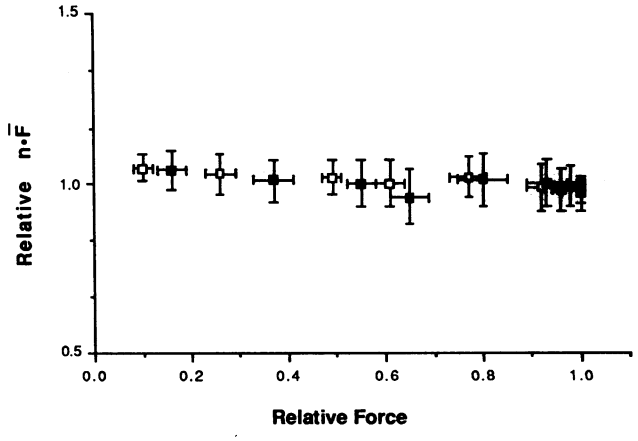

FIG. 4. Calculated values of $n\overline{F}$ versus fraction of prephosphorylation force at pCa 4.3. The data were obtained from 10 fibers before (□) and after (■) myosin light chain phosphorylation. Bars denote SEM.

product $n\bar{F}$ and force. As can be seen, whether or not fibers are phosphorylated, $n\bar{F}$ is relatively constant over the full range of isometric force—i.e., calcium activation levels. This implies that phosphorylation has little or no affect on the number of cycling cross-bridges but primarily affects f_{app}.

DISCUSSION

The results of this study indicate that myosin light chain phosphorylation in skeletal muscle manifests its effect on isometric force and the rate of isometric force redevelopment through a common mechanism, namely increasing the rate at which cross-bridges enter force-producing states from non-force-producing states. Further g_{app}, the rate at which cross-bridges leave the force-producing states, and the number of cycling cross-bridges are unaffected by myosin light chain phosphorylation. Metzger *et al.* (16) demonstrated that myosin phosphorylation alters K_{redev} and thus f_{app}, g_{app}, or both. However, the maximal shortening velocity of rabbit psoas fibers is unaffected by myosin phosphorylation (7, 14). Since maximal shortening velocity is thought to be limited by cross-bridge detachments, it follows that myosin phosphorylation does not affect g_{app} under maximal velocity conditions. However, from this one cannot deduce if g_{app} under isometric conditions is affected by phosphorylation since the values of g_{app} are different during maximal velocity and isometric contractions and a different step in the lumped g_{app} may be rate-limiting.

The effect of myosin light chain phosphorylation on force is maximal at low levels of calcium whereas the maximal effect on K_{redev} is at higher levels of calcium. These facts are consistent with the above and are a consequence of the observation that the ratio of f_{app} to g_{app} is small at low calcium levels and becomes higher as the calcium concentration increases. If $n\bar{F}$ is a constant, then force can be increased by simply increasing the ratio of f_{app} to g_{app} (Eq. 2). At low levels of calcium activation where g_{app} is greater than f_{app}, a small change in f_{app} results in a large change in the fraction of cycling cross-bridges in the force-generating states and thus a relatively large change in force (Fig. 3*B*). As f_{app} becomes large compared to g_{app}, increases in f_{app} have less of an effect on force. When f_{app} becomes much greater than g_{app}, the fraction of cycling cross-bridges in the force-generating states approaches 1. The effect of phosphorylation on K_{redev} can be understood by noting that K_{redev} is simply the sum of f_{app} and g_{app}. When f_{app} is much smaller than g_{app}, a large fractional increase in f_{app} has very little effect on K_{redev} (in fact, K_{redev} approaches g_{app} at the lower levels of calcium activation). When f_{app} is larger than g_{app}, even a small fractional increase in f_{app} results in a significant increase in K_{redev}. Thus the maximal effect of phosphorylation on K_{redev} is found at calcium concentrations greater than the calcium concentration required for the maximal effect on force.

At the lowest levels of calcium activation, K_{redev} approaches g_{app} as force and f_{app} approach zero. The fact that $g_{app} >> f_{app}$ makes it difficult to assign an accurate value to f_{app} since its calculation is critically dependent on the value chosen for g_{app} as well as the accuracy of K_{redev}. Had a slightly larger value of g_{app} been used in calculations, it would have made the calculated value of $n\bar{F}$ greater at low calcium concentrations than at higher ones. Similarly, a smaller value of g_{app} would produce a calculated $n\bar{F}$ that is lower at low calcium concentration than at high concentrations. Assessing whether or not myosin phosphorylation increases force at low calcium concentration by an increase in the fraction of cross-bridges in the force-generating state(s), by increasing the number of cycling cross-bridges, or both is dependent on accurately determining f_{app}. As shown in Fig. 3*A*, the differences in the mean values of f_{app} were within experimental error for control and phosphorylated fibers at the lowest calcium concentrations. Thus, at those concentrations one cannot unambiguously exclude an effect of myosin phosphorylation on $n\bar{F}$. However, it seems unlikely that $n\bar{F}$ would be affected by myosin phosphorylation at low calcium concentrations, but not at higher calcium concentrations.

The effect of calcium on force and K_{redev} (17) is analogous to the effect of phosphorylation; the effect of calcium binding to troponin C changes the value of f_{app}, but g_{app} and the number of cycling cross-bridges are unaffected. Thin-filament regulation exerts primary control over force in striated muscle because when calcium is not bound to troponin C, f_{app} is much smaller than g_{app}. Graded increases in f_{app} occur as calcium binds to troponin C until, at saturating calcium levels, f_{app}/g_{app} is large. Phosphorylation can increase the value of f_{app} at any calcium level thereby enabling modulation of force production and the rate of isometric force redevelopment. Since the enzymes involved in myosin phosphorylation and dephosphorylation are present in skeletal and cardiac muscle, this modulation may serve beneficial roles in locomotion by skeletal muscle and in pumping by the heart.

These results on the increase in f_{app} with myosin light chain phosphorylation may be of fundamental importance for understanding regulation in actin–myosin systems. Whether a system is regulated by thin-filament or thick-filament processes, the control over force may be by changing the value of f_{app}. In other words, relaxation is the consequence of f_{app} being much smaller than g_{app} so that the fraction of cross-bridges in the force-generating states is either very small or zero (if f_{app} is zero). Maximal force output results when f_{app} is much larger than g_{app} and the fraction of cycling cross-bridges in the force-generating states becomes high. If this hypothesis is correct, then myosin light chain phosphorylation may initiate force development in smooth muscle in a manner similar to modulation of force in striated muscle. The f_{app} of smooth or nonmuscle myosin would be near zero in the absence of myosin light chain phosphorylation and larger than g_{app} with phosphorylation. However, because f_{app} for nonphosphorylated myosin in striated muscle is high compared to g_{app} in the absence of inhibition by the troponin–tropomyosin system, force production in striated muscle is high whether myosin is phosphorylated or not. This difference in the properties of striated muscle myosin presumably could have coevolved with thin-filament regulation, which offers the advantage of a much greater rate of activation than could be achieved by phosphorylation. An experimental approach similar to the one presented above may yield interesting insights if applied to the problem of regulation of force by myosin phosphorylation in permeable smooth muscle fibers.

Physiologically myosin light chain phosphorylation will increase the rate and maximal amount of tension developed in skeletal muscle. Under kinetic conditions where the myoplasmic Ca^{2+} concentrations do not saturate the thin-filament regulatory system (single twitches to unfused tetanus responses) (26), repetitive stimuli lead to activation of myosin light chain kinase and myosin light chain phosphorylation (10–13). This phosphorylation will increase the rate of tension production as well as the tension-time integral about 2-fold (10–13, 27). Thus an increase in the intrinsic rate of contractile force generation of fibers in a motor unit will complement the increased rate of force development associated with the recruitment of faster motor units (27, 28).

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