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Regulation of isometric force and isotonic shortening velocity by phosphorylation of the 20,000 dalton myosin light chain of rat uterine smooth muscle

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Abstract. The regulation of isometric force maintenance and isotonic shortening velocity by phosphorylation of the 20,000 dalton light chain of myosin has been examined for potassium-depolarized rat uterine smooth muscle. Following a transient peak in myosin light chain (LC_{20}) phosphorylation at 20 s of contraction (0.46 mol PO_4/mol LC_{20}), phosphorylation declined to a steady-state by 2 min (0.28 mol PO_4/mol LC_{20}) with no significant change from 2–90 min of contraction. Isometric force developed more slowly, reaching a maximum at 2 min with no further change out to 90 min. Lightly-loaded (0.1 F_0) shortening velocity, like LC_{20} phosphorylation, increased initially to a peak of 0.034 L_0/s at 20 s of contraction and then declined to 0.023 L_0/s by 2 min. However, unlike LC_{20} phosphorylation and isometric force, shortening velocity decreased approximately 4-fold from 0.023 L_0/s at 2 min to 0.006 L_0/s at 90 min of contraction. Graded activation with reduced extracellular calcium was associated with proportional changes in steady-state isometric force and LC_{20} phosphorylation. Shortening velocity was also decreased with reduced calcium, however, unlike LC_{20} phosphorylation, the greatest changes in velocity occurred at low levels of developed force. Moreover, in contrast to the large reductions in shortening velocity observed during 90 min contractions where force and LC_{20} phosphorylation were unchanged, similar reductions in shortening velocity did not occur with graded activation in spite of significant (> 3-fold) decreases in both force and LC_{20} phosphorylation. These results suggest that factors other than light chain phosphorylation are involved in the regulation of isotonic shortening velocity during extended isometric contractions of uterine smooth muscle.

Key words: Smooth muscle – Myosin light chain – Phosphorylation – Contraction – Myometrium – Smooth muscle mechanics

Introduction

Phosphorylation of the smooth muscle 20,000 dalton myosin light chain (LC_{20}) has been shown to be a prerequisite for the contraction of avian and mammalian smooth muscle from a number of tissues [6, 7, 19, 25]. The mechanism by which LC_{20} phosphorylation regulates contraction and relaxation, however, remains controversial. One hypothesis is that LC_{20} phosphorylation regulates the number of force producing crossbridges. If it is assumed that smooth muscle contracts via a sliding filament mechanism [23], then this hypothesis could be tested by examining the relationship between LC_{20} phosphorylation and steady-state isometric force at different levels of activation. Experiments of this nature performed with chemically-skinned smooth muscles have demonstrated a correlation between LC_{20} phosphorylation and steady-state isometric force maintenance [8, 19]. Other experiments with skinned smooth muscles have established that LC_{20} phosphorylation and steady-state isometric force are causally related. Calmodulin antagonists, which prevent the phosphorylation of LC_{20}, have been reported to block contraction in the presence of calcium and calmodulin [21]. Irreversible thiophosphorylation of LC_{20} has been shown to prevent relaxation of contracted muscle in the presence of EGTA [7]. It has also been shown that phosphorylation of LC_{20} by a proteolytically activated form of myosin light chain kinase stimulates isometric force development and maintenance in the absence of calcium [29]. These results are all consistent with the hypothesis that LC_{20} phosphorylation regulates the number of attached, force-generating crossbridges and that calcium alone is not able to directly stimulate force development.

Experiments with intact smooth muscle have been more difficult to interpret and have lead some to propose that LC_{20} phosphorylation may regulate the rate of crossbridge cycling while calcium directly regulates the binding of the myosin crossbridge to actin [2]. This hypothesis provides for the formation of attached, noncycling crossbridges if dephosphorylation occurs in the presence of sufficient calcium to prevent dissociation of the myosin crossbridge from actin. The major experimental observations in support of this hypothesis are: 1. changes in LC_{20} phosphorylation and isometric force development have been reported to dissociate during isometric contraction and relaxation [1, 2, 12, 13, 28]; 2. isometric force maintenance has been observed, during both contraction and relaxation, when LC_{20} phosphorylation was not significantly elevated from that measured in relaxed muscles [2, 28]; and, 3. time dependent and calcium dependent changes in unloaded shortening velocity have been reported to correlate with similar changes in LC_{20} phosphorylation [1, 2, 12, 13]. In addition, a recent report based on experiments with skinned vascular smooth muscle indicates that force maintenance during partial relaxation does not require LC_{20} phosphorylation [9].

The current study was designed to investigate the dependence of isometric force maintenance and unloaded shortening velocity on LC_{20} phosphorylation using a uterine

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smooth muscle preparation. Changes in LC_{20} phosphorylation were measured as a function of contraction time and extracellular calcium and then compared to concurrent changes in isometric force maintenance and lightly-loaded (0.1 F_0) shortening velocity. Because correlative observations do not demonstrate causality, particular emphasis was placed on experiments which demonstrated a dissociation of LC_{20} phosphorylation from isometric force or shortening velocity during steady-state conditions.

Methods

Uterine smooth muscle preparation

The longitudinal smooth muscle layer from the uterus of estrogen-primed Sprague-Dawley rats (diethylstilbesterol, 300 μg, ip; 48 and 24 h before use) was dissected free of overlying endometrium and circular muscle. The longitudinal muscle layer was approximately 50–100 μm thick as determined by transmission electron microscopy [14]. For measurements of LC_{20} phosphorylation, strips of muscle (10 mm long × 5 mm wide × < 0.1 mm thick) were attached to Grass FT-0.03 isometric tension transducers and superfused with physiologic saline solution containing 137 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 10 mM $CaCl_2$, 1.2 mM KH_2PO_4, 15.5 mM $NaHCO_3$, and 11.5 mM dextrose. All solutions were continuously bubbled with 5% CO_2 and 95% O_2 (pH 7.4) and maintained at 21 ± 0.5 °C. For measurements of isotonic shortening velocity, strips of muscle were attached to stainless steel pins using a cyanoacrylic adhesive. One pin was attached to a stationary anchor and the other to the movable arm of an electromagnetic ergometer.

The muscles were preconditioned by contraction (10 min) using potassium depolarization (100 mM KCl) in the presence of 10 mM calcium followed by relaxation (10 min) in physiologic saline with no added calcium and 1 mM EGTA. During repeated contractions, the muscle length was adjusted so that the resting tension was maintained at 20% of the total developed force. This procedure was continued until successive contractions showed no change in resting force or total force production. This preconditioning protocol ensured maximum activation of the muscles [14] and established a resting muscle length (L_0) which was at or near the optimum for force production. EGTA (1 mM) was included in the relaxation buffer to eliminate myogenic activity and to ensure complete relaxation. Relaxation of muscles for 10 min in EGTA had no effect on the maximum force production of subsequent contractions.

At appropriate times during the contraction, the muscles were frozen by immersion in dry ice-cooled acetone (−78 °C) containing 10% w/v trichloroacetic acid. A comparison of several freezing techniques showed no significant difference in phosphorylation levels for muscles frozen in liquid nitrogen-cooled isopentane or freon, liquid nitrogen-cooled metal tongs, or dry ice-cooled acetone (data not shown). The frozen muscles were then allowed to warm to room temperature in the acetone-trichloroacetic acid solution to dehydrate the muscles and denature myosin kinase and phosphatase activity. Following three successive washes in acetone containing 1 mM DTT, the muscles were dried and stored at −70 °C. Thawing the muscles in acetone plus trichloroacetic acid has been previously reported to eliminate all myosin light chain kinase and phosphatase activity in crude homogenates of whole frozen muscle, whereas, significant phosphatase activity was still present when muscles were thawed in acetone alone [15]. Inclusion of DTT in the room temperature acetone baths has been found necessary to insure the absence of LC_{20} modification. We have observed that LC_{20} charge modification may result in multiple satellite bands on IEF and urea-glycerol gels which can produce significant errors in the measurement of the stoichiometry of phosphorylation [16].

Measurement of myosin light chain phosphorylation

The stoichiometry of LC_{20} phosphorylation was determined using the radioimmunoblot procedure described in detail elsewhere [17]. Briefly, 100–250 μg of acetone dried muscle was extracted at room temperature for 1 h in 8 M urea (pH 8.6). The supernatant was electrophoresed on urea-glycerol gels to separate the unphosphorylated and phosphorylated forms of LC_{20}. The proteins were then electrophoretically blotted onto nitrocellulose paper. The nitrocellulose segments containing the light chains were then incubated for 1 h in 25 mM Tris HCl (pH 7.5), 150 mM NaCl, and 5% bovine serum albumin at 37 °C. The segments were then incubated overnight at 22 °C in the same buffer containing antiserum for the 20,000 dalton myosin light chain at a dilution of 1:500. The incubation with antiserum was terminated with the following washes at 22° C: 1. 30 min in 25 mM Tris HCl (pH 7.5) and 150 mM NaCl (Buffer A), 2. 80 min in Buffer A containing 0.5% Nonidet P-40 and 5% bovine serum albumin with 1 change, and 3. 30 min in Buffer A alone. The segments were then incubated for 6 h in Buffer A containing 5% bovine serum albumin and [^{125}I] protein-A (0.25 μCi/ml). This incubation was terminated with a series of washes identical to those which followed the antiserum treatment. The portions of the nitrocellulose paper containing each of the two forms of LC_{20} were located by autoradiography and counted in a gamma counter. The stoichiometry of LC_{20} phosphorylation was calculated as the counts associated with the phosphorylated form of LC_{20} divided by the total counts associated with both forms of LC_{20}.

Measurement of isotonic shortening velocity

Isotonic shortening velocity was measured using a servo-controlled electromagnetic ergometer (Model 303, Cambridge Technology). At appropriate times during the contraction, muscles were clamped to an afterload of 10% of the developed active force (F_0) and allowed to shorten isotonically. Lightly-loaded shortening velocity was determined by extrapolating the length change which occurred between 1 and 2 s after the release back to the time of the release as described by Dillon et al. [11].

Statistical methods

All averaged data are reported as mean ± S.E. (n), where n is the number of muscles. Comparison of mean values was by one-way analysis of variance or Student's t-test as indicated in the text.

Results

The time courses of isometric force development, LC_{20} phosphorylation, and lightly-loaded shortening velocity

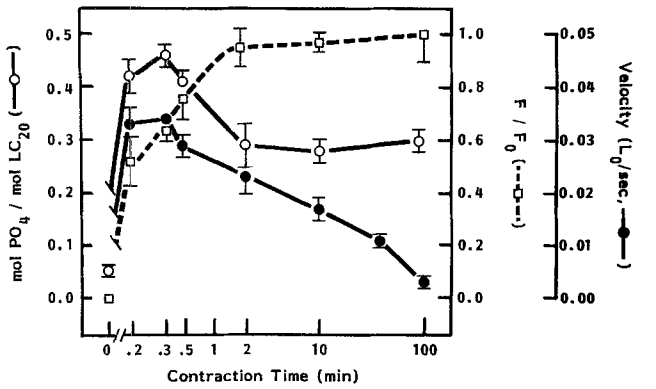

Fig. 1. Time course of isometric force development, LC_{20} phosphorylation, and lightly-loaded shortening velocity for K^+-depolarized rat uterine smooth muscle. Muscles were contracted in the presence of 10 mM extracellular calcium. Isometric force values represent the measured force (F) present at the time the muscles were frozen for phosphorylation analysis divided by the maximum force developed (F_0) during the previous 10 min contraction. Isotonic shortening velocity was measured at an afterload of 0.1 F_0. Shortening velocity is expressed as muscle lengths (L_0) per second. Contraction time is expressed on a logarithmic scale. Values are mean ± S.E. for 3–4 muscles

were determined for potassium-depolarized uterine smooth muscle as shown in Fig. 1. The relative time courses of force development and LC_{20} phosphorylation observed in this study were very similar to data reported by others for potassium-depolarized tracheal and vascular smooth muscle [1, 2, 10, 28] and electrically stimulated taenia coli [4]. LC_{20} phosphorylation peaked by about 20 s of contraction at 0.46 ± 0.021 [4] mol PO_4/mol LC_{20} and then declined to 0.28 ± 0.036 [3] mol PO_4/mol LC_{20} by 2 min. Isometric force development was much slower and did not reach a maximum (0.27 ± 0.023 [5] × 10^5 N/m^2) until approximately 2 min of contraction. Both LC_{20} phosphorylation and isometric force had achieved steady-state values by 2 min. After 90 min of contraction, LC_{20} phosphorylation remained elevated at 0.30 ± 0.015 [6] mol PO_4/mol LC_{20} which was significantly greater than the control value of 0.05 ± 0.007 [3] mol PO_4/mol LC_{20} determined for relaxed muscle. Analysis of variance indicated no significant difference in the phosphorylation or isometric force measured at 2, 10, and 90 min of contraction. Moreover, phosphorylation levels were significantly ($P < 0.05$, Student's t-test) elevated from control throughout the entire contraction. During the early portion of the contraction, up to about 2 min, LC_{20} phosphorylation and shortening velocity had similar time courses. However, unlike isometric force and LC_{20} phosphorylation which remained constant, shortening velocity fell monotonically from a peak of 0.034 ± 0.0010 [7] L_0/s at 20 s of contraction to a minimum of 0.006 ± 0.0013 [3] L_0/s at 90 min of contraction. The change in shortening velocity between 2 and 90 min of contraction was significant ($P < 0.001$) by analysis of variance and represented almost a 4-fold change in shortening velocity during a period when both LC_{20} phosphorylation and isometric force were unchanged. Moreover, the time dependent reduction in shortening velocity was reversible on subsequent contractions. The shortening velocity measured for 10 min contractions which followed a 90 min contraction were 0.87 ± 0.038% [3] of the value measured for 10 min contractions which preceded a 90 min contraction.

Table 1. Comparison of the stoichiometry of myosin light chain phosphorylation at 10 and 90 min of contraction with and without an isotonic quick-release. Muscles were contracted in the presence of 10 mM extracellular calcium. The quick-release was to an afterload of 0.1 F_0. Muscles were frozen within 3 s after the velocity measurement. Values are reported as mean ± S.E. (n), where n is the number of muscles. There was no significant difference among the four means as determined by one-way analysis of variance

Contraction time (min)	Isotonic quick-release	LC_{20} phosphorylation (mol PO_4/mol LC_{20})
10	–	0.26 ± 0.026 (3)
10	+	0.30 ± 0.021 (3)
90	–	0.30 ± 0.027 (4)
90	+	0.29 ± 0.006 (3)

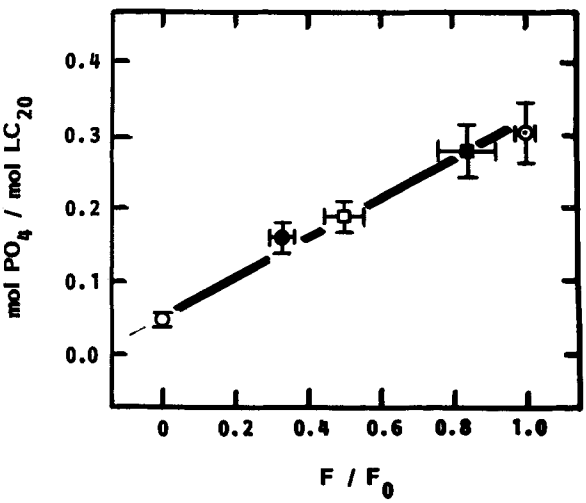

Fig. 2. Gradation of isometric force and LC_{20} phosphorylation with changes in extracellular calcium. The concentrations of extracellular calcium used were: (○), 0 mM + 1 mM EGTA; (●), 0.1 mM; (□), 0.2 mM; (■), 1.0 mM; and (⊙), 10 mM. All muscles were preconditioned by repeated contractions in the presence of 10 mM calcium as described under Methods. Following a 10 min relaxation in the presence of 1 mM EGTA, muscles were contracted by K^+-depolarization in the presence of 0.1–10 mM calcium for 10 min and then frozen for phosphorylation analysis. Isometric force is expressed as a fraction of the maximum active force developed during the previous 10 min contraction in the presence of 10 mM extracellular calcium. Values are mean ± S.E. for 4–6 muscles

To control for the possibility that the quick-release used to measure shortening velocity was itself inducing a change in LC_{20} phosphorylation, phosphorylation measurements were made on several muscles immediately following a quick-release. Muscles were frozen within 3 s of the velocity measurement. The results are summarized in Table 1. Statistical comparisons of the stoichiometries of LC_{20} phosphorylation measured with and without a prior quick-release at both 10 and 90 min of contraction indicated no significant differences.

Having demonstrated that shortening velocity may change in the face of constant LC_{20} phosphorylation, we then manipulated LC_{20} phosphorylation to see if shortening velocity and isometric force were affected. Figure 2 demonstrates that gradation of developed isometric force by changes in extracellular calcium concentration (0.1–

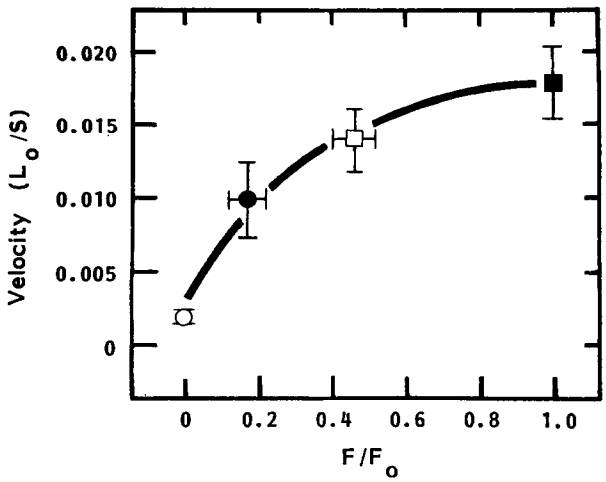

Fig. 3. Gradation of isometric force and lightly-loaded shortening velocity with changes in extracellular calcium. The concentrations of extracellular calcium were: (○), 0 mM + 1 mM EGTA; (●), 0.1 mM; (□), 0.2 mM; and (■), 10 mM. Muscles were contracted as described in Fig. 2. Values are mean ± S.E. for 3–4 muscles

10 mM) produced significant and proportional changes in both isometric force and LC_{20} phosphorylation ($P < 0.05$, analysis of variance) under steady-state conditions (i.e., 10 min of contraction). Shortening velocity was also observed to decrease with reduced extracellular calcium, however, the largest changes in shortening velocity occurred at relatively low levels of active force (Fig. 3). Furthermore, even when isometric force had been reduced to 0.19 F_0, corresponding to a stoichiometry of LC_{20} phosphorylation of < 0.15 mol PO_4/mol LC_{20} (see Fig. 2), shortening velocity was still significantly higher (0.01 ± 0.001 [4] mol PO_4/mol LC_{20}) than that measured after 90 min of contraction (0.006 ± 0.0013 [3] L_0/s).

Discussion

Although it is now generally accepted that LC_{20} phosphorylation is necessary for the contraction of vertebrate smooth muscle, controversy still exists concerning the mechanism by which phosphorylation initiates contraction. One possible mechanism is that LC_{20} phosphorylation stimulates the binding of the myosin crossbridge to actin. If a regulatory system of this nature were present, then there should be a definite relationship between the stoichiometry of LC_{20} phosphorylation and the steady-state stress produced by a contracting muscle at any defined muscle length. This relationship would not necessarily be linear. For example, in vitro experiments with smooth muscle actomyosin suggest that both heads of the myosin molecule must be phosphorylated to stimulate actin-activated myosin ATPase activity [24, 26]. Moreover, the two heads are phosphorylated sequentially, resulting in a non-linear relationship between myosin phosphorylation and ATPase activity [24, 26]. Any such correlation between force and phosphorylation, however, would only necessarily be apparent under steady-state conditions. If the kinetics of LC_{20} phosphorylation were significantly faster than those of force development, there could be dissociation of these two events under nonsteady-state conditions. The results of the work presented here indicate a positive correlation between steady-state isometric force and LC_{20} phosphorylation which is consistent with the hypothesis that LC_{20} phosphorylation regulates the number of attached, force generating crossbridges.

An alternate mechanism by which LC_{20} phosphorylation could regulate contraction has been suggested by Murphy and his co-workers [2]. The basic hypothesis is similar to one which has been previously described for certain invertebrate smooth muscles. Molluscan smooth muscles are able to maintain developed force, isometrically, for long periods of time with very low rates of ATP hydrolysis [20]. This highly efficient force maintenance is possible because crossbridge detachment during relaxation may be very slow depending on the nature of the stimulus. The existence of similar "noncycling crossbridges" or "latch bridges" in vertebrate smooth muscle has been previously hypothesized based on mechanical [5, 22] and energetic [4, 27] measurements. This idea has received renewed attention recently as a result of the suggestion that LC_{20} phosphorylation may regulate the isotonic shortening velocity of smooth muscle [2]. It has been demonstrated that shortening velocity closely parallels the actin-activated myosin-ATPase activity for a wide range of muscle types [3]. Assuming that the sliding filament model of muscle contraction applies to smooth muscle, changes in LC_{20} phosphorylation should correlate with changes in shortening velocity if LC_{20} phosphorylation regulates the enzymatic activity of the myosin ATPase. As discussed above, such a relationship between shortening velocity and LC_{20} phosphorylation would not necessarily be linear. The most critical test of this hypothesis would be the observation that either LC_{20} phosphorylation or shortening velocity changed while the other remained constant.

The experiments reported here for uterine smooth muscle suggest that the relationship between isometric force, shortening velocity, and LC_{20} phosphorylation may be different than that reported for carotid artery [1, 2, 13] and trachealis [12] smooth muscle. The correlation between steady-state isometric force maintenance and phosphorylation observed in the current study is consistent with the hypothesis that phosphorylation regulates the number of force producing crossbridges in uterine smooth muscle. We have reported elsewhere that two mechanistically different agonists, carbachol and oxytocin, produce time courses of isometric force development and LC_{20} phosphorylation which are similar to those reported in the current study with phosphorylation remaining significantly elevated throughout the 90 min contractions [17]. In general we have never observed a dissociation between isometric force and LC_{20} phosphorylation under steady-state conditions in uterine smooth muscle. In contrast, it has been reported in carotid [1, 2, 12] and trachealis [12, 28] smooth muscle that isometric force may remain elevated while phosphorylation decreases or returns to basal values during tonic contractions.

The current study also demonstrates that LC_{20} phosphorylation and isotonic shortening velocity do not show a consistent correlation during steady-state contraction as has been reported for carotid [1, 2, 13] and trachealis [12] smooth muscle. During 90 min contractions there was a clear dissociation of LC_{20} phosphorylation from shortening velocity which suggests that a factor other than LC_{20} phosphorylation, acting either alone or in conjunction with LC_{20} phosphorylation, modulates isotonic shortening velocity. In contrast to this time dependent dissociation, graded

activation resulted in reductions of both shortening velocity and phosphorylation at 10 min of contraction. Although this latter observation could be interpreted as support for the hypothesis that phosphorylation regulates cross-bridge cycling rate at 10 min of contraction, this would not be consistent with the observed dissociation of these two parameters during longer contractions. An equally plausible hypothesis, that a time-dependent internal resistance to shortening is present in uterine muscle, would be consistent with both observations. Such an internal resistance would presumably cause the greatest reductions in shortening velocity at low levels of activation, resulting in a non-linear relationship between shortening velocity and developed force similar to that which was observed in the current study. The presence of such an internal resistance, in the form of attached, noncycling crossbridges, has previously been suggested by others [4, 5, 22, 27].

In conclusion, the data presented here support the hypothesis that phosphorylation regulates the number of attached, force-generating crossbridges in uterine smooth muscle. Moreover, the clear dissociation of shortening velocity and phosphorylation observed during prolonged contractions indicates that factors other than, or in addition to, phosphorylation are involved in the regulation of isotonic shortening velocity.

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