Cross-bridge regulation by Ca$^{2+}$-dependent phosphorylation in amphibian smooth muscle

C. J. WINGARD,¹,² J. M. NOWOCIN,¹ AND R. A. MURPHY¹
¹Department of Physiology, Medical College of Georgia, Augusta, Georgia 30912; and ²Department of Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

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Wingard, C. J., J. M. Nowocin, and R. A. Murphy. Cross-bridge regulation by Ca$^{2+}$-dependent phosphorylation in amphibian smooth muscle. Am J Physiol Regulatory Integrative Comp Physiol 281: R1769–R1777, 2001.—A covalent regulatory mechanism involving Ca$^{2+}$-dependent cross-bridge phosphorylation determines both the number of cycling cross bridges and cycling kinetics in mammalian smooth muscle. Our objective was to determine whether a similar regulatory mechanism governed smooth muscle contraction from a poikilothermic amphibian in a test of the hypothesis that myosin regulatory light chain (MRLC) phosphorylation could modulate shortening velocity. We measured MRLC phosphorylation of Rana catesbiana urinary bladder strips at 25°C in tonic contractions in response to K$^+$ depolarization, field stimulation, or carbachol stimulation. The force-length relationship was characterized by a steep ascending limb and a shallow descending limb. There was a rapid rise in unloaded shortening velocity early in a contraction, which then fell and was maintained at low rates while high force was maintained. In support of the hypothesis, we found a positive correlation of the level of myosin phosphorylation and an estimate of tissue shortening velocity. These results suggest that MRLC phosphorylation in amphibian smooth muscle modulates both the number of attached cross bridges (force) and the cross-bridge cycling kinetics (shortening velocity) as in mammalian smooth muscle.

MAMMALIAN SMOOTH MUSCLE differs from skeletal muscle because both the extent of cross-bridge recruitment (determining steady-state force) and the kinetics of cross-bridge cycling (manifested by unloaded shortening velocities or power output) vary with the level of activation (18). This difference is not due to the myosin motors, although the smooth muscle isoforms have a low ATPase activity, but is conferred by differences in the regulatory mechanisms. In skeletal muscle, Ca$^{2+}$ acts allosterically by binding to a thin-filament regulatory protein, troponin. By contrast, the primary regulatory mechanism in mammalian smooth muscle is covalent. Ca$^{2+}$ binds to calmodulin, and this complex activates myosin light chain kinase (MLCK). Phosphorylation of Ser¹⁹ of the myosin regulatory light chain (MRLC) by MLCK allows the cross bridge to bind to the thin filaments and cycle. Thus Ca$^{2+}$-dependent phosphorylation determines cross-bridge recruitment.

While myosin light chain phosphatase (MLCP) activity can be regulated, it is considered constitutively active in mammalian smooth muscle in the simplest regulatory paradigm for steady-state activation. When myoplasmic Ca$^{2+}$ concentrations are elevated, the kinetics of cross-bridge phosphorylation and dephosphorylation are comparable to those of cross-bridge cycling. This leads to a situation in which both phosphorylated and dephosphorylated cross bridges contribute to force development (18). However, the kinetics of detachment differ between the phosphorylated and dephosphorylated cross bridges, leading to differences in cycling rate (5). Thus phosphorylation also determines shortening velocities. Physiologically, this system is advantageous because it allows comparatively rapid phasic contractions and also the slowing of cycling rates and ATP consumption during sustained tonic contractions when muscle in the walls of hollow organs serves a structural role in stabilizing organ dimensions against imposed loads.

There is limited information on the mechanics and contractile behavior of isolated frog stomach cells (2, 23, 27, 28) and the pharmacology of teleost and reptile vascular smooth muscle (17, 30) and frog bladder smooth muscle (3, 12, 31). However, we found no measurements of cross-bridge phosphorylation or information on whether ectothermic vertebrate smooth muscle exhibits “latch” (i.e., maintained force in the presence of low MRLC phosphorylation and cross-bridge cycling).

Our objectives were to see whether Ca$^{2+}$-dependent MRLC phosphorylation triggers contraction in an amphibian smooth muscle and whether cross-bridge cycling rates are regulated by this mechanism, in a test of the hypothesis that MRLC phosphorylation regulates shortening velocity.

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MATERIALS AND METHODS

Tissue preparation. All animal care and use was conducted in accordance with National Institutes of Health guidelines and a protocol approved by the Medical College of Georgia and the University of Virginia Animal Care and Use Committees. Urinary bladders were removed from Rana catesbeiana after anesthesia with MS-222 and euthanized by double pithing. Two 3- to 4-mm-wide strips were dissected from each hemisphere of the bladder. Each strip was folded over on itself, and the ends were secured in an aluminum foil clip with cyanoacrylate to form a ring.

All bladders were bathed either in a bicarbonate-based Harris amphibian Ringer solution (ARS) at 25°C, bubbled with a 20% oxygen-75% nitrogen-5% CO2 mix, and adjusted to pH 7.4, or in a MOPS-based physiological saline solution (PSS) also adjusted to a pH of 7.4 at 25°C and bubbled with air. The ARS contained (in mM) 90.4 NaCl, 2.9 KCl, 0.48 MgSO4, 30.0 NaHCO3, 1.2 Na2HPO4, 3.0 d-glucose, and 1.5 CaCl2. The MOPS-based buffer contained (in mM) 140.0 NaCl, 5.0 KCl, 1.6 CaCl2, 1.2 MgCl2, 1.2 Na2HPO4, 5.6 d-glucose, 2.03 MOPS, and 0.02 EDTA (to chelate trace metals). The use of both buffers resulted in similar force-generating capacities of the bladder strips in response to K+ depolarization or carbachol stimulation. The MOPS-based buffer was used for all mechanics and myosin phosphorylation experiments for its ability to allow storage of tissues overnight in the cold without continued gassing to maintain the pH.

Spontaneous contractions were seen in <20% of all tissues examined. When spontaneous contractions occurred, the CaCl2 concentration in either solution was lowered to 0.8 mM and then returned to 1.5 or 1.6 mM in the potassium-containing solutions (K+-ARS or K+-PSS). Lowering of Ca2+ concentration had previously been shown to reduce or eliminate spontaneous contraction (21). The K+-ARS or K+-PSS solutions contained stoichiometrically substituted KCl for NaCl. If preparations displayed oscillations during agonist stimulation, the steady-state force was estimated by fitting a straight line through the trace.

During field stimulation, 0.5 mM ascorbic acid was added to all solutions to protect against free radical formation. A Protech (JDR Microdevices, San Jose, CA) sweep function generator, model B-801, provided 2- to 20-Hz direct current square-wave pulses delivered through two platinum foil electrodes to the bladder rings and a force transducer (Astro-Med/Gould Instruments Division, West Warwick, RI) containing springs allowing force measurements up to 200 g. The lower post was attached to a Grass FT 03 force transducer (Astro-Med/Astro-Med, East Bloomfield, NY) (0.01-mm resolution). The compliance of the system was primarily due to transducer displacement, and a correction factor was applied to all reported data. Force was recorded on a Gould eight-channel recorder (model 8800, Gould Instruments Systems, Valley View, OH). All force measurements were converted into stress values (force/cross-sectional area) (29). Cross-sectional area of tissues at L0 was calculated from the tissue measurements of length, mass, and density (area = (wt weight/length at L0)/(1.055 g/cm3)).

Mounted rings were equilibrated for 30 min in ARS or PSS at a length at which there was <2 g of resting force. The rings were then stretched using several protocols to determine the force-length relationship. Initially, rings were stretched using three 0.5-mm increments with intervening stress relaxation for 8–15 min. After the third stretch, a 0.5-mm quick release was imposed (Fig. 1A). The passive force (Fp) was estimated as the minimal force after the release before any tone redevelopment. Variations in the stretching protocol included larger 0.75-mm incremental stretches with a smaller 0.25-mm release, or a single 1.0-mm stretch followed by three 0.5-mm stretches with a 0.5-mm quick release. Only after the release and force stabilization at the new length were the preparations stimulated. The tissues were depolarized (73 mM K+) for a minimum of 3 min and relaxed in ARS or PSS for a period of 15 min. The next increment of stretches was performed only when resting force in PSS equaled the prestimulus resting force. The protocol was repeated until forces fell to 0.5 peak values or the tissues became too long to remain immersed in the bath. Fp and total force (Ft) at each length were measured.

Active force (Ft - Fp) was calculated after correction for apparatus compliance. The active force-length data for individual preparations were fitted by a third-order polynomial (29). The choice of this function was based on the best statistical fit (typically r2 > 0.9) and has no theoretical significance. The equation allowed a reference length (L0) to be identified for each preparation (where L0 = L when dF/ dL = 0). Normalized force or stress values are reported as the measured value (F or S, respectively) divided by the calculated value of F0 or S0, respectively, where F0 = F and S0 = S at L0.

Microscopy. Several rings were fixed for histological examination in 1.5% glutaraldehyde/0.05 M sodium cacodylate buffer at L0 for 2 h to determine the orientation and fractional content of smooth muscle cells. The tissues were then washed in 0.05 M sodium cacodylate buffer and rinsed in veronal acetate. The tissues were stained en bloc with 3% uranyl acetate in veronal acetate for 2 h. After washing in veronal acetate, the tissues were dehydrated in increasing percentages of 70–100% ethanol, followed by two washes in 100% acetone. The tissues were then infiltrated with an acetone-Poly/Bed 812 mix (Polysciences, Warrington, PA). After two additional changes of pure Poly/Bed 812 resin, the tissues were embedded in the resin at 60°C for 2 days. Thick sections were cut with a LKB III ultramicrotome (Leica, Deerfield, IL). The sections were dried on glass slides and stained with toluidine blue.

Shortening velocity. Shortening velocities were calculated from the estimated time to force redevelopment after releases of 10, 12.5, and 15% of tissue length during K+ depolarization at L0. The times for force redevelopment for a set of releases at 5, 10, 20, 30, 60, and 90 s into a contraction were fitted by linear regression. The slope of the regression represents the unloaded shortening velocity, and the Y-intercept provides an estimate of the series elasticity. Fits were done on individual tissues, and a mean value was calculated for all preparations. Only those fits that returned r2 values of >0.85 were included in the subsequent analysis.

Cross-bridge phosphorylation. Phosphorylation determinations were made in separate tissues under identical treatment conditions because of the destructive nature of the assay. A dry ice-acetone slurry (–78°C) was used to freeze rings after treatments at selected time intervals. The rings were slowly thawed in acetone over a 2-h period, air dried, weighed, and homogenized in a cold aqueous solution containing 1% (wt/vol) SDS, 10% glycerol (vol/vol), and 20 mM diithiothreitol (7). Segments of the tissue secured and glued with cyanoacrylate were discarded before homogenization.
Phosphorylation of the smooth muscle-specific 20-kDa MRLC isoform was determined by two-dimensional isoelectric focusing (IEF) and SDS-PAGE (7). Dephosphorylated and phosphorylated myosin light chains were separated in the first dimension by IEF using an ampholyte gradient of pH 4–6.5 (Pharmacia LKB Technology, Piscataway, NJ). Myosin light chains were then separated from other proteins by molecular weight in the second dimension using SDS-PAGE. The gels were stained with colloidal Coomassie blue (ICN Biomedicals, Aurora, OH). Phosphorylation values were determined densitometrically using a BioImage 2000 digital image system (Bio Image, Ann Arbor, MI) and a Sun Sparc 10 computer.

Alternatively, MRLC phosphorylation was determined using a one-dimensional IEF slab gel protocol (4) with an ampholyte gradient of pH 4–6.5. The separated proteins in the slab gels were then transferred to either 0.22-μm nitrocellulose or polyvinylidene difluoride (PVDF) membranes (Micro Separations, Westborough, MA) via a tank blotter (Hoff Scientific, San Francisco, CA). Immunolabeling of the smooth muscle-specific MRLC isoform was accomplished using a monoclonal anti-MRLC raised against turkey gizzard MRLC (20 kDa) clone MY-21 (Sigma, St. Louis, MO), 1:1,000 dilution in 0.1% Tween-Tris-buffered saline. Detection used a 1:2,000 dilution of a goat anti-mouse IgM-specific peroxidase-conjugated secondary antibody. Protein was detected using a 3,3′-diaminobenzidine-based colorimetric substrate, digitally scanned for density and analyzed using ImageQuant software. All phosphorylation values are reported as the density of the monophosphorylated MRLC relative to the density of the total amount of MRLC isoform detected, from the following equation: %MRLC-P<sub>i</sub> = (phosphorylated band density)/ (unphosphorylated band density + phosphorylated band density)) × 100.

Statistical analysis. Data were analyzed using ANOVA for repeated measures with post hoc comparisons made by Student-Newman-Keuls test. Student's t analysis was used where appropriate. Statistical significance was set at P < 0.05.

RESULTS

Preparations of urinary bladder from R. catesbiana contracted in response to electrical field stimulation, carbachol stimulation, and K<sup>+</sup> depolarization. These contractions were sustainable for more than 3 min and displayed a fast rising peak force component and a slower sustained force component.

Physical properties of urinary bladder strips. Force in the urinary bladder rings depends on tissue length (Fig. 1). In response to 73 mM K<sup>+</sup> depolarization, the peak $F_p$, length-tension relationship of individual preparations could be approximated by a third-order polynomial, and a theoretical maximal force and optimal length for force generation ($L_o$) could be identified (29) (Fig. 1B). The average force-length relationship for the urinary ring preparation had a flat plateau (0.9–1.1 $L_o$) and a very shallow descending limb (1.1–1.7 $L_o$) (Fig. 1C) compared with mammalian smooth muscle (29) (Table 1).

To ensure that the bladder preparations were oriented in their proper direction for monitoring force generation, representative strips were fixed at $L_o$ and sectioned for light microscopy. Cross-sectional and longitudinal sections revealed extensive connective tissue...
Table 1. Comparison of properties of frog bladder, mammalian bladder, and swine carotid

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Stimulus (Temperature)</th>
<th>Maximal Stress, $10^5$ N/m²</th>
<th>Ascending and Descending Slope, relative stress/relative length</th>
<th>Shortening Velocity, cm/sec</th>
<th>SEC, % of L₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rana bladder</td>
<td>73 mM K⁺ (25°C)</td>
<td>0.437</td>
<td>1.07 and −0.32</td>
<td>0.303</td>
<td>5.6</td>
</tr>
<tr>
<td>Rabbit bladder</td>
<td>K⁺ depolarization (37°C)</td>
<td>1.3</td>
<td>ND</td>
<td>0.29</td>
<td>3–4</td>
</tr>
<tr>
<td>Swine carotid</td>
<td>109 mM K⁺ (37°C)</td>
<td>1.76,† 1.23‡</td>
<td>1.28 and −1.36‡</td>
<td>0.12¹</td>
<td>7.2‡</td>
</tr>
</tbody>
</table>

SEC, series elastic component; L₀, reference length. Cited from *Ref. 26, †Ref. 10, and ‡Ref. 29. ND, not determined.

and nonmuscle cells. Smooth muscle occurred in discrete bundles in these sections (Fig. 2A). The higher magnification of longitudinal sections revealed smooth muscle cells aligned at a small angle to the vector of force measurements. (Fig. 2B). The fractional content of the smooth muscle cells of these preparations was small, composing only $10.1 \pm 8.5\%$ ($n = 4$) of cross-sectional area. The basic physical characteristics of the urinary ring preparations, including wet weight, cross-sectional area at L₀, and contribution of $F_p$ to K⁺-dependent force, are reported in Table 2.

Stimulus-response behavior. Unstimulated bladder preparations occasionally showed spontaneous contractile activity. This was abolished or diminished by reducing the bathing Ca²⁺ concentration from 1.6 to 0.8 mM (data not shown). Reduced CaCl₂ did not affect the subsequent stress-generating ability of the tissue in response to K⁺ depolarization, carbachol, or field stimulation, provided that 1.6 mM Ca²⁺ was present in the stimulation solution and that time was given to restore Ca²⁺ stores between stimulations.

The phasic component of the contractile response to K⁺ depolarization had an EC₅₀ of 17.5 mM K⁺ and was maximal above 30 mM K⁺. The same behavior was seen for the steady-state stress response (Fig. 3A). The presence of 0.5 μM tetrodotoxin to block neural effects before and during stimulation resulted in a small increase in peak and steady-state stress generation with K⁺ depolarization (Fig. 3B, B and C). However, this small increase in stress was not statistically significant in paired comparisons ($P > 0.125$).

The contractile responses of the urinary bladder to carbachol were biphasic with increasing levels of peak and steady-state stress generation occurring over the concentration range of 0.01–1.0 μM (Fig. 4A). Concentrations greater than 100 μM significantly reduced both the peak and steady-state stress responses (Fig. 4B). The EC₅₀ for carbachol was 6.4 nM.

The response of Rana bladder to field stimulation was strongly biphasic (Fig. 5). When we used a fixed 20-Hz stimulation frequency, the peak and steady-state stress response increased steeply with increased current. Peak stress occurred with 60 mA of current with a threshold of 30 mA (Fig. 5B). The dependence of stress on stimulus frequency was biphasic, having a significantly larger peak stress value in the first 30 s of a stimulus. Subsequently, stress declined to the lower steady-state values (Fig. 5A). The peak stress values were constant over a 2- to 20-Hz range and somewhat lower at the highest frequencies (Fig. 5C). However, the steady-state stress levels fell with increasing frequency of stimulation.

MRLC phosphorylation and cross-bridge cycling. MRLC phosphorylation was initially determined using the two-dimensional gel technique for separation of protein based on their IEF point and then by their molecular weight (Fig. 6A). The Rana bladder and the swine carotid media exhibited similar major protein

Table 2. Physical properties of the Rana bladder preparation subjected to 73 mM K⁺ depolarization

<table>
<thead>
<tr>
<th></th>
<th>Wet Weight, mg</th>
<th>Cross-Sectional Area at L₀, mm²</th>
<th>Peak Stress at L₀, $10^5$ N/m²</th>
<th>$F_p/F₀$ at L₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>27.8</td>
<td>1.796</td>
<td>0.437</td>
<td>0.351</td>
</tr>
<tr>
<td>SE</td>
<td>±3.1</td>
<td>±0.187</td>
<td>±0.050</td>
<td>±0.026</td>
</tr>
<tr>
<td>n</td>
<td>63</td>
<td>62</td>
<td>50</td>
<td>50</td>
</tr>
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$F_p$, passive force; $F₀$, total force.
compositions in gels stained with Coomassie blue. However, proteins with mobilities characteristic of the nonmuscle MRLC isoforms were absent in the frog bladder, which is also true for mammalian visceral and urogenital smooth muscle (1, 7).

Detection of MRLC was also accomplished by Western blots of two dimensions using a monoclonal anti-mouse IgG antibody labeling the smooth muscle myosin light chain spots, identified as SM-MLC. We also used a simplified gel preparation where homogenates of the urinary bladder were loaded in lanes of an IEF slab gel and run overnight under identical conditions used to run the first dimension of the two-dimensional system. The gel was then transferred to either nitrocellulose paper or PVDF membrane and exposed to the MRLC antibody (Fig. 6B). The one-dimensional IEF and transfer system for MRLC detection provided reproducible results and similar quantitative results to that reported by the two-dimensional system. We report here quantitative changes in light chain phosphorylation by the two-dimensional Coomassie blue detection because of limitations in the detection linearity of Western reaction products. MRLC phosphorylation levels rose from an unstimulated value of 11.6 ± 1.5% to a peak of 50.9 ± 1.1% in 20 s of stimulation with 73 mM K⁺ (Table 3). Phosphorylation then fell to 26.7 ± 1.2% by 60 s and remained at ~25% for the remainder of a stimulation (up to 3 min, data not shown).

**Unloaded shortening velocities.** Cross-bridge cycling kinetics as estimated from unloaded shortening velocity ($V_{\text{us}}$) measurements were dependent on MRLC phosphorylation. In individual bladder preparations the slack times after 10, 12.5, and 15% length releases were fitted with a linear regression. The slope of this regression-estimated shortening velocity and the intercept provided an estimate of the series elastic shortening in a K⁺-induced contraction. The slack times for 10, 12.5, and 15% length releases during a K⁺ depolarization at 5, 30, and 90 s are shown in Fig. 7B. The fits revealed an initial slope at 5 s, which progressively steepened, peaking at 30 s, and then fell to a slope shallower than the initial 5-s determination at 90 s. The calculated mean $V_{\text{us}}$ normalized for tissue lengths is reported in Table 3. The mean $V_{\text{us}}$ exhibited a linear dependence on the level of MRLC phosphorylation with a slope of ~0.008 $L_0$/s per %MRLC phosphorylation.

**Fig. 3.** Stress generation of *Rana* bladder rings during graded K⁺ depolarization. A: representative force trace from a bladder ring preparation submaximally stimulated with KCl without TTX. B: peak active stress to graded K⁺ concentration in presence and absence of 0.5 µM TTX. C: steady-state active stress in bladder rings stimulated with K⁺ in presence and absence of 0.5 µM TTX. Values are presented as means ± SE (n = 6 for peak value determinations and 8 for steady-state determinations). *Statistically significant difference from value reported at 73 mM K⁺ (P < 0.025).

**Fig. 4.** Active stress generation of *Rana* bladder rings stimulated with carbachol. A: representative force traces for a cumulative carbachol dose response of a bladder ring preparation. B: peak and steady-state stress responses are reported for graded carbachol stimulation. Values are presented as means ± SE (n = 4). *Statistically significant difference from value reported at 1 µM carbachol (P < 0.001).
These results also provided an estimate of the mean series elasticity of 5.9 ± 0.8% at the maximal force generated (Table 3).

The time-dependent changes in stress generation, shortening velocity, and MRLC phosphorylation are shown in Fig. 8. Stress rose monotonically, achieving a plateau in 30 s. The averaged data did not show the typical initial phasic peak (Fig. 8) due to variability reflected by the large SE (dashed lines). Statistical analysis of mean data showed no differences in stress values between the 20-, 30-, 60-, and 90-s time points (see Table 3). Increases in MRLC phosphorylation were correlated with increases in stress and $V_{usc}$. However, stress was sustained while MRLC phosphorylation and $V_{usc}$ fell. This phenomenon of force maintenance with reduced cross-bridge cycling rates as manifested by $V_{usc}$ is behavior termed “latch” in mammalian smooth muscle.

DISCUSSION

The frog urinary bladder exhibited contractile properties similar to those of mammalian smooth muscle tissues, including latch at 25°C. This mechanical behavior was correlated with changes seen in MRLC phosphorylation responsible for the activation of the smooth muscle cross bridge. Both observations support
the hypothesis that cross-bridge phosphorylation can regulate tissue shortening and contractile behavior in *Rana* bladder.

The micrographs of the bladder strips confirmed previously described cellular arrangements in the urinary bladder (20). There was a relatively thick stromal layer containing collagen, blood vessels, and smooth muscle bundles bounded by mucosal and serosal epithelial layers. The alignment of the smooth muscle cells in the preparations was reasonable, if not optimal, for mechanical measurements.

The dependence of force on length was measured using a protocol developed for the swine carotid media (29). Our results are consistent with described force-length relationships for mammalian bladder preparations (6, 16, 26). The bladder strips display a broad range of lengths around $L_0$ where there is little change in the active stress (Fig. 1 and Ref. 8). This behavior may be attributed to anatomic arrangements of the smooth muscle bundles resulting in different bundles becoming optimized for force generation at different lengths. The descending limb of the force-length relationship was relatively shallow compared with skeletal and mammalian smooth muscles. This observation may also reflect the anatomic arrangement of the muscle bundles. The biphasic stress-length relationship is consistent with a sliding filament/cross-bridge paradigm (9) although correlation with filament overlap is lacking in any vertebrate smooth muscle.

The maximal force response to most stimuli was usually biphasic with a peak generated within 60 s followed by a slow decline to a steady-state level. Such a response is typical for most mammalian urinary bladder preparations (13–15). The sustained contractile response to $K^+$ depolarization with or without an initial transient (Figs. 3 and 8) is characteristic of a tonic smooth muscle (24).

The series elastic component (SEC) was estimated to be $6\%$ in $K^+$-depolarized tissues, generating around $3.0 \times 10^4$ N/m$^2$ (Table 3). This value is not significantly different from that reported for the rabbit bladder (26). This SEC value does not reflect the SEC of the cross bridge itself but is a refection of the ensemble SEC of the tissue preparation, and its accuracy is limited by the method used to fit slack times. The maximal short-
erking velocity of 0.30 $L_0/s$ was also comparable to a variety of mammalian bladder preparations (26). The mechanical performance of the $Rana$ bladder strips at 25°C is remarkably similar to the reported behavior of mammalian bladder at 37°C.

Phosphorylation of the MRLC is accomplished by the Ca$^{2+}$-dependent action of MLCK and is reversed by MLCP. Recent evidence suggests that equivalent changes in MLCK and MLCP activities seen at different temperatures should have a minimal impact on cross-bridge recruitment or phosphorylation kinetics (22). Thus we anticipated a minimal impact on the relationship between myosin phosphorylation, isometric force generation, and shortening velocity for the frog bladder at room temperature compared with a mammalian preparation at 37°C. The maximal force generation in mammalian smooth muscle is only slightly affected by change in temperatures from 22 to 37°C and only moderately affected over the temperature range of 10–22°C (8, 11, 19). However, the shortening velocities and the rates of force generation and relaxation are more highly temperature dependent. The conclusions drawn from these studies were that the temperature dependence of the ADP release step, as well as the temperature dependence of the regulatory system, are likely responsible for the differences (11). If the in situ temperature dependency of MLCK and MLCP in amphibian smooth muscle were the same, one would predict that lowering temperature would not increase net MRLC phosphorylation in response to a specific stimulus-induced elevation in myoplasmic Ca$^{2+}$. Several studies on the effect of moderate cooling on contractile response in mammalian smooth muscle have demonstrated that the hypersensitivity is related to an increased release of intracellular Ca$^{2+}$ and alteration in the electrogenic Na$^+/K^+$ exchanger, resulting in a general increase in intracellular Ca$^{2+}$ concentration (25). Such a rise in Ca$^{2+}$ would lend itself to an increased MLCK activity and MRLC phosphorylation. Because $V_{is}$ is directly proportional to MRLC phosphorylation (Fig. 7), this would offset any slowing due to the temperature dependence of cross-bridge cycling.

The behavior of the amphibian bladder at 25°C in response to a muscarinic agonist, depolarization, and field stimulation was quantitatively similar to mammalian bladder at 37°C. Our data support the hypothesis that Ca$^{2+}$-dependent cross-bridge phosphorylation is responsible for activation in response to excitatory stimuli. As in mammals, high forces can be sustained with moderate elevations in MRLC phosphorylation and with reduced cross-bridge cycling rates as estimated from $V_{is}$ in the frog bladder preparation at room temperature.

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