Myoplasmic [Ca\(^{2+}\)] Determines Myosin Phosphorylation in Agonist-Stimulated Swine Arterial Smooth Muscle

Christopher M. Rembold and Richard A. Murphy

Our objective was to test the hypotheses that 1) myoplasmic [Ca\(^{2+}\)] is the primary determinant of crossbridge phosphorylation and that 2) phosphorylation is the primary determinant of crossbridge interactions with the thin filament in swine carotid arterial smooth muscle. We tested these hypotheses by evaluating the relation between aequorin-estimated myoplasmic [Ca\(^{2+}\)], myosin light chain phosphorylation, shortening velocity at zero load (V\(_o\)), and stress at various times after stimulation with histamine, phenylephrine, and depolarization with KCl. Agonist-induced changes in myoplasmic [Ca\(^{2+}\)] were associated with predictable changes in myosin phosphorylation. Depolarization required proportionally higher changes in myoplasmic [Ca\(^{2+}\)] for a given change in myosin phosphorylation. The relation between phosphorylation and V\(_o\) or steady-state stress was invariant with all tested stimuli. This suggests that Ca\(^{2+}\)-dependent crossbridge phosphorylation is the primary determinant of the mechanical response. (Circulation Research 1988;63:593–603)

There is persuasive evidence that [Ca\(^{2+}\)]-stimulated myosin phosphorylation is a key event in the regulation of crossbridge cycling in smooth muscle (reviewed by Kamm and Stull!). Nevertheless, other Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent processes have been proposed to be involved in the regulation of contraction as well. An important step in the assessment of these regulatory mechanisms is to determine whether changes in the second messenger (Ca\(^{2+}\)) determine changes in crossbridge phosphorylation during contraction and relaxation in smooth muscle. This criterion was satisfied in one case, in which aequorin-estimated myoplasmic [Ca\(^{2+}\)] was correlated with myosin phosphorylation and mechanical estimates of crossbridge interactions in depolarized swine carotid media. Several investigators have reported estimates of myoplasmic [Ca\(^{2+}\)] that were not directly proportional to force. Such results were interpreted as showing that other regulatory systems may be involved in contraction of smooth muscle. However, these reports lacked phosphorylation measurements and the relation between myosin phosphorylation and steady-state stress is reportedly hyperbolic. This suggests that the relation between [Ca\(^{2+}\)] and stress may not be linear.

This study addresses two questions: 1) are agonist-induced changes in myoplasmic [Ca\(^{2+}\)] the sole determinant of crossbridge phosphorylation; and 2) do changes in crossbridge phosphorylation determine the mechanical response? An affirmative answer to the first question would support the hypothesis that agonist-induced changes in [Ca\(^{2+}\)] and subsequent activation of myosin light chain kinase are the major factors determining myosin phosphorylation. Invariant relations between myosin phosphorylation and active stress or shortening velocity at zero load (V\(_o\)) would support the hypothesis that crossbridge phosphorylation may be sufficient to explain the mechanical response. Our approach was to correlate estimates of myoplasmic [Ca\(^{2+}\)], myosin phosphorylation, V\(_o\), and active stress in swine carotid media stimulated by several agonists.

**Materials and Methods**

**Tissue Preparations and Aequorin Loading**

Swine common carotid arteries were obtained from a slaughterhouse and transported at 0°C in physiological salt solution (PSS). Dissection of...
medial strips, mounting, and determination of the optimum length for stress development were performed as described by Driska et al.11 The intimal surface was mechanically rubbed to remove the endothelium. PSS consisted of (mM) NaCl 140, KCl 5, 3-[N-morpholino]propanesulfonic acid (MOPS) 2, CaCl₂ 1.6, MgCl₂ 1.2, Na₂HPO₄ 1.2, d-glucose 5.6 (pH adjusted to 7.4 at 37° C). Depolarization was accomplished by substituting 10 or 20 mM KCl stoichiometrically for NaCl in PSS. Solution changes and depolarization were performed by draining the organ bath by a vacuum line and refilling via tubes through light-tight traps. Agonist stimulation was accomplished by rapid injection of an appropriate volume of 10 mM stock histamine or phenylephrine into the tissue bath. Stock solutions of agonists were prepared daily. Tissues that did not produce a stress greater than 1.0 x 10⁵ N/m² after 109 mM KCl depolarization at 37° C or develop 50% of the final stress after 2 minutes of depolarization were discarded (see “Results”).

Aequorin (batch 2, obtained from Dr. John Blinks, Mayo Medical School, Rochester, Minnesota) was loaded by a modification of the method of Rembold and Murphy, which was based on that of Morgan and Morgan.12 The modification was to omit the determination of the optimal length for stress development (L₀) prior to the loading procedure. This change avoided a warming and cooling cycle and resulted in a marked decrease in basal light production with a greater aequorin loading efficiency. Arterial strips were dissected in 2°C PSS and incubated free-floating at 2°C in the following sequence of solutions (mM): 1) 20 minutes in ethylene glycol bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) 10, KCl 120, ATP 5; MgCl₂ 2; N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) 20 (pH 6.8 at 2°C); 2) 90 minutes in aequorin 0.01, EGTA 0.1, KCl 120, ATP 5; MgCl₂ 2; 3) 30 minutes in EGTA 0.1, KCl 120, ATP 5, MgCl₂ 10, TES 20 (pH 6.8); and 4) 30 minutes in NaCl 140, KCl 5, MgCl₂ 10, MOPS 2, glucose 5.6 (pH 7.1 at 22°C). The tissues were then mounted isometrically, stretched to a length that produced a stress of approximately 0.5 x 10⁵ N/m², and warmed to 22°C. CaCl₂ was added to give the following concentrations at 15-minute intervals: 0.001, 0.01, 0.1, and 1.6 mM. The arterial strips were incubated in PSS with 100 U/ml aqueous penicillin G and 100 μg/ml streptomycin overnight and were warmed to 37°C during the night by use of a timer in the heating water circulator circuit. The next morning, the tissues were stretched to a length producing approximately 1.0 x 10⁵ N/m² passive stress, and then shortened to reduce the passive stress to 0.1–0.2 x 10⁵ N/m². The final length was within 5% of L₀. Experiments were begun after an equilibration period (≥60 minutes) and antibiotic washout.

Light Detection

Light measurements were made in a light-tight enclosure on the apparatus of Morgan and Morgan and Blinks as described previously.5 The apparatus was modified by connecting the photomultiplier output to a C-10 photon counter (Thorn EMI, Plainview, New Jersey). The analog output was filtered (usually with a 0.1–1 second time constant) and displayed on a Linseis Model 2045 rectilinear recorder (Princeton Junction, New Jersey). The dark count of the photomultiplier tube was collected for at least 60 seconds and subtracted from all subsequent counts. Typical dark counts were between 60 and 100 counts per second, and the dark count was rechecked during and after contractions. The dark count did not rise when the shutter was opened if the chamber did not contain a tissue or if an unloaded preparation was mounted in the apparatus. We concluded that basal light production (typically 200–500 counts per second) reflects the light emission by aequorin in unstimulated preparations.

Calibration

Aequorin light signals are presented in the form log L/Lₘₐₓ, where L is the photon count (in counts per second) and Lₘₐₓ is an estimate of the peak light intensity that would be recorded if all of the aequorin in the tissue was instantaneously exposed to 5 mM CaCl₂. Lₘₐₓ can be considered a measure of the total undischarged aequorin present in the tissue. Hence, the ratio L/Lₘₐₓ will be invariant with respect to the efficiency of aequorin loading in different tissues. Lₘₐₓ was calculated according to the method of Allen and Blinks.14 At the end of each experiment, hypotonic 5 mM CaCl₂ at 37°C was slowly added to lyse the cells and the light production recorded (slow addition produced lower peak light counts and thus decreased the likelihood of coincident photons that would serve to underestimate the true Lₘₐₓ). The photon count was recorded until all the aequorin was discharged (typically, this required 15–30 minutes of incubation). The total photon count collected during and at the end of an experiment is also a measure of the initial undischarged [aequorin] in the tissue. Lₘₐₓ, the peak light intensity that would be recorded if a similar amount of aequorin was instantaneously exposed to 5 mM CaCl₂, was calculated from the total photon count by means of the equation: Lₘₐₓ = total photon count/0.4 sec. This equation relates the total photon count to the peak light intensity by means of a “time constant,” which was determined to be 0.4 second at 37°C (D.G. Allen, personal communication). No errors are introduced by variations in the value of the time constant since the same value was used both in the calibration and the experimental measurements.

The number of photons (in counts per second) was averaged over 10-second periods during the first 5 minutes of contraction and over 60-second
periods at later times. The ratio \( L/L_{\text{max}} \) was calculated by subtracting the previously determined dark count from the average count and dividing by \( L_{\text{max}} \) (which was calculated at each time point). The \( \log_{10} \) of \( L/L_{\text{max}} \) was then calculated. Averaging decreased the effect of photomultiplier noise and allowed more accurate estimation of myoplasmic \([\text{Ca}^{2+}]\).

Tissues were weighed after incubation in 5 mM \( \text{CaCl}_2 \), which may produce osmotic swelling. The regression line comparing the weights of 12 tissues before and after incubation in 5 mM \( \text{CaCl}_2 \) for 30 minutes at 37° C had a slope of 1.02 (\( r = 0.98 \)). No weight correction was made in the calculation of tissue cross-sectional area and stress values.

Aequorin was calibrated using an apparatus constructed from an Aminco photometer head attached to a temperature-controlled aluminum block. This apparatus has a diaphragm that allows light-tight injection of aequorin using a Hamilton constant-rate syringe (Reno, Nevada). An Aminco fluo-calorimeter was used to amplify the photomultiplier output (Silver Spring, Maryland). Ten microliters of \( 1 \mu \text{M} \) aequorin (preequilibrated with 0.5 mM \( [\text{Mg}^{2+}] \) at 37° C) was injected into 1 ml of a \( \text{Ca}^{2+}\)-EGTA buffer with (mM) KCl 120, TES 20, EGTA 1, and \( \text{MgCl}_2 \) 0.5 (pH 7.1 at 37° C). The following protocol was used to ensure that the desired \([\text{Ca}^{2+}]\) were obtained in the calibration solutions. A 0.1 M \( \text{CaCl}_2 \) solution was gravimetrically prepared by adding HCl to \( \text{CaCO}_3 \). A standard curve of \([\text{Ca}^{2+}]\) (made with dilutions of \( \text{CaCl}_2 \) in 120 mM KCl and 20 mM TES, pH 7.1 at 37° C) versus millivolts was generated with an Orion 93-20 \( \text{Ca}^{2+} \) electrode (Boston, Massachusetts). Then, six different mixtures of the \( \text{CaCl}_2 \) and a nominally 0.1 M EGTA solution (Sigma E 4378, pH 7.1 with KOH) were prepared in the same KCl/TES solution, and the \( \text{Ca}^{2+} \) activities were measured with the \([\text{Ca}^{2+}]\) electrode. Scatchard analysis was performed and the true [EGTA] determined. Stock 0.01 M EGTA and 0.01 M CaEGTA solutions were made using the calculated [EGTA] and the pH adjusted to 7.1 at 37° C. The amount of CaEGTA and EGTA added to the \( \text{Ca}^{2+}\)-solutions was calculated with a multiple-equilibrium computer program.

Eleven different solutions with \([\text{Ca}^{2+}]\) in the range 0.1 to 10 \( \mu \text{M} \) were prepared and the light production measured. A calibration curve of \( \log L/L_{\max} \) versus pCa was then plotted. This relation was fitted with the sigmoideal curve in the form \( y = y_0/(1 + (K_{30}/x)^N) \). This curve was obtained by least squares fitting of the transform \( y' = \log y/(y_0 - y) \) and \( x' = \log x \). The slope of the transform is \( N \) and the y intercept is \(-N \log K_{30} \). In this calibration, \( x = \text{pCa}, \ y = \log L/L_{\max} \), and \( y_0 \) is the log \( L/L_{\max} \) observed in EGTA (i.e., \( \text{Ca}^{2+} \)-independent light production = -6.2). We derived a calibration equation of \( \log L/L_{\max} = -6.2(1 + (6.011/\text{pCa})^2.356) \) at \( [\text{Mg}^{2+}] = 0.5 \) mM. The r value was 0.996 with \( n = 82 \). The goodness of fit was estimated by inspecting the residual of each measurement; that is, the model predicted \([\text{Ca}^{2+}]\) (based on \( \log L/L_{\max} \) change) was compared with the solution \([\text{Ca}^{2+}]\) (as determined by the ratio

\[ \frac{\text{Estimated Basal } \text{[Ca}^{2+}\text{]} \text{ nM}}{50} \times 500 \]

\[ \frac{\text{Basal log } L/L_{\max}}{50} \times 500 \]

\[ \text{Maximal Active Stress} \times 10^5 \text{ N/m}^2 \]

\[ \text{r} = 0.88 \]

\[ \text{n} = 32 \]

\[ \text{FIGURE 2.} \text{ The relation between maximal stress produced (note log scale) and basal log } L/L_{\max} \text{ in 32 consecutive preparations that were stimulated with 109 mM KCl or 10 } \mu \text{M histamine (these are near maximal stimuli for the preparation). The dashed line represents the cutoff for acceptance of the experiment and is based on the stress attained.} \]
of Ca²⁺ to EGTA). The percentage difference between the model predicted [Ca²⁺] and the solution [Ca²⁺] was 11.3 ± 1.0% (n = 82) in the [Ca²⁺] range 0.1 to 10 μM and by 12.5 ± 1.6% (n = 44) in the [Ca²⁺] range 0.1 to 0.6 μM.

The calibration with 0.5 mM [Mg²⁺] was selected to reflect the estimated [Mg²⁺] in this tissue. Some investigators may feel this is a low estimate of [Mg²⁺]. If the [Mg²⁺], was assumed to be 1.25 mM, the relation log L/Lₘₐₓ = -6.2/(1 + (5.8/pCa)⁽⁴⁻²¹⁷⁾) can be used to recalculate the estimates of [Ca²⁺] given in the text.⁵

Myosin Phosphorylation

Myosin light chain phosphorylation in tissues frozen by immersion at -78°C was determined by the method of Driska et al.¹¹ (with precautions noted by Aksoy et al.).⁶ Phosphorylation is reported as moles inorganic phosphate (Pᵢ) per mole total 20 kD light chains. The minor satellite species were ignored.¹¹ Phosphorylation was determined both in control tissues and in tissues that underwent a "sham" aequorin loading procedure.

Mechanical Measurements

Force was measured isometrically with a Grass FT.03 (Quincy, Massachusetts) force transducer in both the light detection apparatus and for determination of phosphorylation. The compliance of the system was 4.2 μm/g. Force was displayed on the rectilinear recorder in synchrony with the light signal. Stress was calculated as force per cross-sectional area, which was estimated from measured length, weight, and a density of 1.050 g/cm³. Active stress was determined as the difference between total stress and the passive stress that was determined by quick release to the optimal length for stress development (see section on aequorin loading).

Shortening velocity at zero load was estimated using isotonic quick-releases to varied afterloads.¹⁷ This involved a different apparatus based on a Cambridge Technology (model 300H, Cambridge, Massachusetts) dual mode servo that was interfaced to a Northstar Horizon microcomputer (San Leandro, California). Software (Peter Becker, © ALETA) allowed storage of length and force after releases to fractional loads. Isotonic shortening velocity for a given load was calculated by fitting an exponential curve to tissue length at 200 time points from 1 to 2 seconds after release.¹⁸ Velocities were measured at four or five loads (Fᵣ) ranging from 0.1–0.5 F₀ (the isometric load before the release). A plot of (1-Fᵣ/F₀)/velocity versus Fᵣ/F₀ was drawn and the least-squares regression line calculated. The V₀ was estimated as the y intercept of the regression line, and experiments were accepted if r exceeded 0.90.

Statistics

Forces were compared by using Students unpaired t test. Values of p<0.05 were considered significant. All values are given as mean ± SEM.

Results

Evaluation of Aequorin Loading Procedure

We compared the stress generated by the aequorin-loaded preparations with unloaded preparations that were assayed for myosin phosphorylation. Stress development in response to each of the stimuli employed in this study was not significantly impaired by the aequorin loading procedure (Figure 1). The modified aequorin loading procedure increased the [Ca²⁺] detection sensitivity by reducing basal light production. Furthermore, the percentage of successful experiments increased from approximately 25% to 75%. However, tissue damage or deterioration resulted in lower stress production in some preparations. Tissues were accepted for measurement of [Ca²⁺] or myosin phosphorylation only if maximal stress production (defined as that attained during stimulation with 10 μM histamine or 109 mM KCl) was greater than 1.0 x 10¹³ N/.
This level of stress was 58–69% of the stress normally developed by this tissue with these stimuli (Figure 1). We evaluated the resting [Ca\(^{2+}\)] in 32 consecutive preparations that were maximally stimulated with 10 \(\mu M\) histamine or 109 mM KCl depolarization. Those preparations with higher basal light production produced lower stress (Figure 2, \(p<0.01\)). This finding is consistent with tissue damage and the need for stress generation criteria.

The efficiency of the modified aequorin loading procedure was evaluated. The active intracellular aequorin concentration at the beginning of a contraction was estimated by dividing \(L_{\text{max}}\) for an arterial strip by both the \(L_{\text{max}}\) of a known concentration of aequorin and the tissue’s cell volume (estimated by weight, assuming a density of 1.05 g/cm\(^3\) and a previously determined cell fraction (60%, Murphy et al\(^{19}\)). We measured \(8.7 \times 10^6\) counts/pmole aequorin in our apparatus (based on the aequorin supplier’s determination of [aequorin]). In 16 preparations, the mean estimate for the cellular concentration of active aequorin was \(7.1 \pm 0.8\) nM. This yielded a loading efficiency, defined as the intracellular concentration of active aequorin divided by the concentration of aequorin in loading solution 2, of 0.071%.

**Calibration of Light Signals**

The major goal of this study was the correlation of estimates of myoplasmic [Ca\(^{2+}\)] with levels of myosin phosphorylation and mechanical activation. Definitive calibration is impossible with any indicator due to uncertainties in both the intracellular environment and the cellular localization of the indicator. Calibrations can only be performed in solutions approximating cellular temperature, ionic strength, pH, [Mg\(^{2+}\)], etc. The most important determinant of aequorin light production besides [Ca\(^{2+}\)] is [Mg\(^{2+}\)]. We performed calibrations with [Mg\(^{2+}\)] equal to 0.5 mM (see ‘Materials and Methods’) based on estimates from nuclear magnetic resonance\(^{15}\) and microelectrode studies (H. Yamaguchi, personal communication). The conclusions of this study are based on a direct comparison of \(\log L/L_{\text{max}}\) with myosin phosphorylation and are independent of the assumptions in the calibration that is provided for comparison with other results.

Basal \(\log L/L_{\text{max}}\) was \(-5.50 \pm 0.03\) \((n=16)\) which corresponds to a basal [Ca\(^{2+}\)] of \(120 \pm 6\) nM based on the calibration in 0.5 mM Mg\(^{2+}\). Previously we estimated basal [Ca\(^{2+}\)] to be approximately 500 nM.\(^3\) Several factors contributed to the lower [Ca\(^{2+}\)] estimates including improved aequorin loading with lower basal light production and a new calibration. The resting [Ca\(^{2+}\)] is more comparable to estimates of resting [Ca\(^{2+}\)] obtained with the fluorescent dyes quin2 or fura-2\(^{20,21}\) and microelectrodes.\(^{22}\) The increase in sensitivity greatly improved the detection of small changes in aequorin estimated [Ca\(^{2+}\)].

**Representative Light Signals**

Figure 3A shows a light and force recording after stimulation with 109 mM KCl. Mean data revealed that aequorin-estimated Ca\(^{2+}\), phosphorylation, and \(V_0\) all reached peak values during the first minute of stimulation and then fell to lower levels while stress increased to a sustained high value (Figure 4). Light peaked at 60 seconds with \(\log L/L_{\text{max}}\) of \(-4.64 \pm 0.04\) (([Ca\(^{2+}\)] \(= 330 \pm 12\) nM). By 30 minutes, \(\log L/L_{\text{max}}\) fell to \(-5.00 \pm 0.04\) (([Ca\(^{2+}\)] = 230 \pm 10 nM). If the level of depolarization was reduced after 3 minutes by switching to 20 mM KCl, then \(\log L/L_{\text{max}}\), phosphorylation, and \(V_0\) were also proportionaly reduced (see Figures 3B and 8). At 30 minutes in 20 mM KCl, \(\log L/L_{\text{max}}\) was \(-5.18 \pm 0.04\) (([Ca\(^{2+}\)] = 190 \pm 9 nM). Substantial stress was maintained with...
development of the latch state characterized by low values of phosphorylation and shortening velocity.

A light transient was observed in response to 10 \( \mu M \) histamine stimulation (Figure 5). Stress developed rapidly, and was maintained at near peak levels despite a fall in light to levels below those observed in both depolarization protocols (Figures 3, 4, and 8). At 30 minutes, log \( \frac{L}{L_{\text{max}}} \) was \(-5.23 \pm 0.04\) ([Ca\(^{2+}\)] = 180 ± 10 nM). This log \( \frac{L}{L_{\text{max}}} \) value was significantly greater than basal log \( \frac{L}{L_{\text{max}}} \) \((p<0.01)\). Calibration was not attempted during the transient because aequorin light signals overrepresent regions with the highest [Ca\(^{2+}\)] and uniformity of [Ca\(^{2+}\)] is less likely during transients. Steady-state light was lower and myosin phosphorylation, \( V_o \), and stress levels were higher with histamine stimulation than with 109 mM KCl depolarization (Figure 4). This suggests that the relation between aequorin-estimated myoplasmic [Ca\(^{2+}\)] and myosin phosphorylation may differ between agonist- and depolarization-induced contractions.

Sequential histamine contractions were performed after varying periods of relaxation (Figure 5). The light transient was large in the initial contraction, with a change in log \( \frac{L}{L_{\text{max}}} \) of 0.98 ± 0.55. The transient was diminished when a second contraction followed 15 minutes of histamine washout (change in log \( \frac{L}{L_{\text{max}}} \) = 0.51 ± 0.18), although this did not reach statistical significance \((p = 0.15, n = 3)\). After a waiting period of 150 minutes, the histamine-induced transient again resembled the transient induced during the first contraction (change in log \( \frac{L}{L_{\text{max}}} \) = 0.88 ± 0.56). The light response at 30 minutes of contraction showed relatively little depen-
Figure 7. Time course of \( L/L_{\text{max}} \), myosin light chain phosphorylation, shortening velocity at zero load, and stress after stimulation with 10 \( \mu \)M histamine (open circles and dashed line) or 10 \( \mu \)M phenylephrine (open squares and dotted line). At the right of the \( L/L_{\text{max}} \) tracing is a calibration for \( [\text{Ca}^{2+}] \), assuming \( M_{\text{g}^{2+}} \) to be 0.5 mM. Bars are mean±SEM (n=4 for \( L/L_{\text{max}} \) and stress, 4–6 for phosphorylation, and 5–6 for velocity).

The relation between aequorin-estimated \( [\text{Ca}^{2+}] \) and myosin phosphorylation was examined by comparing log \( L/L_{\text{max}} \) and myosin phosphorylation after stimulation for 1, 3, 10, or 30 minutes with histamine, phenylephrine, and depolarization (Figure 9). Histamine and phenylephrine both elicited a similar dependence of myosin phosphorylation on myoplasmic \( [\text{Ca}^{2+}] \). In contrast, the \( \text{Ca}^{2+} \)-sensitivity of myosin phosphorylation was shifted approximately a half log unit to the right in depolarized tissues.

Correlation Between Aequorin-Estimated Myoplasmic \( [\text{Ca}^{2+}] \) and Myosin Phosphorylation

The relation between aequorin estimated \( [\text{Ca}^{2+}] \) and myosin phosphorylation was examined by comparing log \( L/L_{\text{max}} \) and myosin phosphorylation after stimulation for 1, 3, 10, or 30 minutes with histamine, phenylephrine, and depolarization (Figure 9). Histamine and phenylephrine both elicited a similar dependence of myosin phosphorylation on myoplasmic \( [\text{Ca}^{2+}] \). In contrast, the \( \text{Ca}^{2+} \)-sensitivity of myosin phosphorylation was shifted approximately a half log unit to the right in depolarized tissues.

Dependence of \( V_0 \) and Stress on Phosphorylation

\( V_0 \) was proportional to myosin phosphorylation over a wide range of phosphorylation values and was independent of the stimulus (Figure 10A). Steady-state stress correlated with changes in myosin phosphorylation over the range of 0.10 to 0.30 mol P/mol myosin light chain (MLC) (Figure 10B). Maximal steady-state stress was attained at approximately 0.30 mol P/mol MLC. The dependence of steady-state stress on myosin phosphorylation was invariant with these different forms of stimulation (Figure 10B).

Discussion

Several investigators reported that estimates of \( [\text{Ca}^{2+}] \) and force production are not always correlated in smooth muscle.6–8 This is not surprising because there are a number of nonlinear intermediate steps between agonist stimulation and stress development. We felt it necessary to address two questions: 1) what is the relation between \( [\text{Ca}^{2+}] \), and myosin phosphorylation, and 2) what is the relation between myosin phosphorylation and the mechanical response?

The second question was clearly answered. The relation between myosin phosphorylation and \( V_0 \) or stress was invariant with agonist stimulation and depolarization. This confirms the report of Ratz and Murphy9 that the relation between phosphorylation
and stress is very steep at low levels of myosin phosphorylation, and that near maximal steady-state stress is produced at levels of myosin phosphorylation over approximately 0.30 mol P/mol MLC. Higher values of phosphorylation accelerate the rate of force development and \( V_0 \). The direct dependence of \( V_0 \) on phosphorylation observed with agonists (Figure 10A) confirms previous studies in the depolarized swine carotid. This behavior can be predicted by a model postulating a noncycling "latchbridge" formed by dephosphorylation of an attached, cycling crossbridge in which \([\text{Ca}^{2+}]\) dependent phosphorylation is the only postulated regulatory process.10

In contrast, the relation between aequorin-estimated myoplasmic \([\text{Ca}^{2+}]\) and myosin phosphorylation varied when agonist-stimulated preparations were compared with depolarized tissues. There are several potential explanations for this observation.

1) The \([\text{Ca}^{2+}]\) sensitivity of myosin light chain kinase (MLCK) could be altered in a stimulus-specific manner. 2) Myosin phosphatase could be selectively regulated by different stimuli. 3) Myosin could be phosphorylated by another kinase in some cases. 4) The aequorin signal may not always reflect the \([\text{Ca}^{2+}]\) which determines MLCK activity. MLCK can be phosphorylated by cyclic AMP (cAMP)-dependent protein kinase in tissues24 and this phosphorylation was associated with decreased \([\text{Ca}^{2+}]\) sensitivity in vitro.25 MLCK would need to be selectively phosphorylated during depolarization-induced contractions to explain the observed response. However, cAMP levels were low and constant throughout depolarization-induced contractions of swine carotid.16,26 Other investigators reported variable changes in cAMP with histamine stimulation. Kolbeck and Spier27 reported that histamine-induced contractions in guinea pig tracheal smooth muscle were associated with a significant decrease in cAMP content after 6 minutes of stimulation, while Peterson28 found that 10 \( \mu \)M histamine elevated cAMP in 150 mM KCl-depolarized swine carotid arteries. Even if cAMP were to change during those contractions, the effect on MLCK activity would be small. Kamm and Stull2 reported that phosphorylation of MLCK would have only a minor effect on the \([\text{Ca}^{2+}]\) sensitivity of MLCK at estimated myoplasmic [calmodulin] and [MLCK]. Thus, large changes in the \([\text{Ca}^{2+}]\) sensitivity of MLCK associated with changes in MLCK phosphorylation are possible, but unlikely.

Pato et al29,30 and DiSalvo and Gifford31 isolated several phosphatases from smooth muscle. Some of these phosphatases (MLCP) were capable of dephosphorylating native myosin, but these were not regulated by \([\text{Ca}^{2+}]\) or \([\text{Mg}^{2+}]\). However, MLCP is po-
agonists could reduce the phosphatase activity. Decrease the level of myosin phosphorylation, or conceivably enhance phosphatase activity and thus change the phosphorylation-stress relation. This was not observed (Figure 10B).

Protein kinase C has been reported to phosphorylate smooth muscle heavy meromyosin at threonine 9 and/or serine 1 or 2. This phosphorylation had no effect on actin-activated ATPase activity per se and decreased the ATPase activity of myosin already phosphorylated by MLCK by 50%. This change in ATPase activity suggests that changes in protein kinase C-dependent phosphorylation would change the relation between myosin phosphorylation and . However, we found no stimulus-dependent shift in the phosphorylation- relation (Figure 10A). This suggests that direct protein kinase C-dependent phosphorylation does not explain the difference in the [Ca2+] phospho-relations observed. We found other actions of kinase C that might modify myoplasmic [Ca2+] or the sensitivity of myosin phosphorylation.

It is also possible that aequorin light production may not represent the true [Ca2+], that regulates MLCK activity. Qualitatively similar time courses of changes in [Ca2+], have been reported with several different indicators. Defeo and Morgan reported that quin2-loaded ferret portal vein cells showed a transient rise in fluorescence in response to phenylephrine and a sustained response to 140 mM KCl. These results were similar to aequorin-estimated [Ca2+] changes obtained by the same investigators in ferret portal vein. Depolarization with 140 mM KCl produced a sustained rise in quin2 fluorescence while 100 µM norepinephrine or 100 µM histamine produced transients followed by a significant fall in fluorescence with maintained stress in helically cut strips of rabbit ear artery. Other investigators reported agonist-dependent Ca2+ transients as measured by quin2 or fura-2. In contrast, depolarization reportedly produced a sustained rise in estimated [Ca2+] as evaluated by quin2 or a Ca2+-selective microelectrode. These studies suggest that all the available techniques predict similar qualitative directional changes in myoplasmic [Ca2+] with comparable stimuli. In addition, Fabiato reported that mechanically skinned Purkinje fibers adsorb and concentrate aequorin. If the smooth muscle myoplasm also concentrates aequorin, then aequorin is more likely to be a myofibrillar compartment indicator.

Inhomogeneities in agonist-receptor density may result in a nonuniform [Ca2+] response in the cell population. However, histamine stimulation produced stress values equal to the highest obtained with the combination of depolarization and elevated extracellular CaCl2. This result implied that most cells responded to histamine. Histamine produced 68% phosphorylation at 1 minute of stimulation, a value that limits the absolute heterogeneity to approximately 30% of the cells with 0% phosphorylation and the remaining cells with 100% phosphorylation. Even this implausible, extreme case of inhomogeneity would not explain the shift in the [Ca2+] phospho-dependencies observed.

Myoplasmic Ca2+ could be compartmentalized during contractions. van Breemen et al hypothesized that a region between the plasma membrane and superficial sarcoplasmic reticulum has a higher [Ca2+] during contractions than the rest of the cytoplasm. They proposed that this [Ca2+] gradient is maintained by the high activity of nearby Ca2+ pumps and the small volume in this space. If this proposed gradient were relatively larger during depolarization, the shift in the [Ca2+] myosin phosphorylation relation would be explained. We found evidence for focal consumption of aequorin with sequential histamine contractions (Figure 5). If focal consumption were occurring during the histamine transient, then it also might occur in the space between the plasma membrane and superficial sarcoplasmic reticulum that is proposed to have higher [Ca2+]. Direct spatial measurements of [Ca2+] in depolarized and agonist-stimulated cells are required to evaluate this potential artifact. One final uncertainty is the effect of intracellular ionic changes.
which occur with prolonged depolarization. These may artifactually shift the [Ca\(^{2+}\)]-myosin phosphorylation relation in depolarized tissues.

In conclusion, 1) agonist-induced changes in [Ca\(^{2+}\)], determined crossbridge phosphorylation and 2) phosphorylation, per se, determined crossbridge cycling rates and steady-state active stress. If this were true for all agonists, our results suggest that Ca\(^{2+}\)-dependent crossbridge phosphorylation would be the only significant regulatory mechanism necessary to explain stress development and maintenance in the swine carotid. A potential exception was the effect of depolarization where higher aequorin-estimated myoplasmic [Ca\(^{2+}\)] was required to induce phosphorylation. This exception may be caused by spatial gradients in [Ca\(^{2+}\)], ionic changes during depolarization, or modulation of the [Ca\(^{2+}\)] dependence of myosin phosphorylation.

Acknowledgments

The authors would like to thank Mildred Smythers for technical assistance. Arteries were donated by Smithfield Co., Smithfield, Virginia.

References

7. DeFeo TT, Morgan KG: Calcium force relationships as detected with aequorin in two different vascular smooth muscles of the ferret. *J Physiol* 1983;369:269–282
31. Matsumoto T, Kanaide H, Nishimura J, Shogakiuchi Y, Kobayashi S, Nakamura M: Histamine activates...
H1-receptors to induce cytosolic free calcium transients in cultured vascular smooth muscle cells from rat aorta. Biochem Biophys Res Commun 1986;135:172-177


37. Fabiato A: Rapid ionic modifications during the aequorin-detected calcium transient in a skinned canine cardiac Purkinje cell. J Gen Physiol 1985;85:189-246


KEY WORDS • activation-contraction coupling • crossbridge phosphorylation • smooth muscle mechanics • crossbridge cycle • calmodulin • catecholamines • histamine
Myoplasmic [Ca2+] determines myosin phosphorylation in agonist-stimulated swine arterial smooth muscle.

C M Rembold and R A Murphy

_Circ Res._ 1988;63:593-603
doi: 10.1161/01.RES.63.3.593

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/63/3/593

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at: http://circres.ahajournals.org/subscriptions/