Myoplasmic Calcium, Myosin Phosphorylation, and Regulation of the Crossbridge Cycle in Swine Arterial Smooth Muscle

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SUMMARY Our objective was to test the hypothesis that changes in crossbridge phosphorylation in the swine carotid media are due to changes in the myoplasmic calcium concentration. The photoprotein aequorin was loaded intracellularly by incubation in a series of calcium-free solutions. This loading procedure did not affect subsequent stress development, myosin light chain phosphorylation, or ultrastructure. The time course of light production, myosin light chain phosphorylation, shortening velocity at zero load, and active stress were measured in three stimulus protocols: depolarization with 109 mM potassium chloride at (1) 22°C, (2) 37°C, and (3) 37°C, followed by a reduction in potassium chloride to 20 mM to induce stress maintenance with basal phosphorylation (latch). Light-predicted intracellular calcium concentration was found to correlate with myosin phosphorylation and unloaded shortening velocity. The calcium concentration required for half-maximal myosin phosphorylation was approximately twice that for stress maintenance. These estimates depend on many assumptions, but they compared favorably with the half-maximal myosin phosphorylation values obtained for the calcium-dependence of stress maintenance and phosphorylation in Triton X-100 skinned carotid media preparations. This supports the hypothesis that myoplasmic calcium is the determinant of myosin phosphorylation and mean crossbridge cycling rates in intact smooth muscle depolarized by potassium chloride (Circ Res 58: 803-815, 1986).

Ca++-CALMODULIN activation of myosin light chain kinase (MLCK) and subsequent phosphorylation of the regulatory light chain of myosin is an important regulatory mechanism in smooth muscle (reviewed by Kamm and Stull, 1985a): (1) Biochemical studies have shown that the myosin regulatory light chains can be stoichiometrically phosphorylated by an endogenous kinase (MLCK) and dephosphorylated by an endogenous phosphatase (MLCP). (2) Myosin phosphorylation is accompanied by a proportional increase in actin-activated myosin adenosine triphosphatase (ATPase) activity in vitro. Furthermore, (3) studies of intact tissues have shown that myosin phosphorylation is required for stress development, and that steady state mean crossbridge cycling rates (estimated by shortening velocities at zero load) are directly proportional to the level of myosin phosphorylation. This body of work meets three of four criteria listed by Krebs and Beavo (1979) which must be satisfied to establish a functional role of protein phosphorylation in cellular regulation. The fourth criterion is to demonstrate that cellular Ca++ concentrations are correlated with the extent of crossbridge phosphorylation. This would establish that inputs to the cell membrane regulate crossbridge phosphorylation and the biological response by altering myoplasmic [Ca++]. The overall objective of this study is to determine whether stimulus-induced changes in myoplasmic [Ca++] produce proportional change in myosin phosphorylation and mechanical activation. To accomplish this objective, a technique for the estimation of myoplasmic [Ca++] is required which meets the following criteria. (1) The method must be applicable to arterial smooth muscle tissues, where the high elastin content and autofluorescence prevent the use of fluorescence indicators. (2) The response of the system must be sufficiently rapid to permit resolution of cellular Ca++ transients (Blinks, 1982). (3) The technique should be relatively unaffected by movement artifacts associated with tissue contraction (Baylor, 1983). (4) The techniques should have a high sensitivity and ideally be capable of accurately quantifying cellular Ca++ levels down to 0.1 μM. Finally (5) the estimates should reflect tissue averages for correlation with estimates of phosphorylation and the mechanical response. Until recently, none of the various approaches to estimating cellular Ca++ concentrations met these criteria. Fay et al. (1979) reported light signals from amphibian stomach smooth muscle cells that were pressure injected with the photoprotein aequorin. This technique is difficult in mammalian smooth muscle; however, the development by Morgan and Morgan (1982, 1984) of a procedure for chemically loading aequorin into smooth muscle cells offers a potentially useful approach. The aequorin technique meets the first three criteria. The major concerns are...
whether the sensitivity of aequorin is sufficiently high, whether the aequorin is effectively distributed in the myoplasmic compartment, and whether the Ca\(^{2+}\) calibration of the light signals is valid.

Myosin light chain phosphorylation and shortening velocities often decrease while stress is maintained in tonic contractions (Dillon et al., 1981; Aksoy et al., 1983; Kamm and Stull, 1985b; Weisbrodt and Murphy, 1985). This has been termed the latch state, and is characterized by force maintenance with reduced ATP consumption (Butler et al., 1983; Krisanda and Paul, 1984). Data from skinned swine carotid media suggest that force maintenance by dephosphorylated crossbridges is Ca\(^{2+}\) dependent (Güth and Junge, 1982), and depends on a second regulatory mechanism which is more sensitive to Ca\(^{2+}\) than the calmodulin-myosin light chain kinase cascade (Chatterjee and Murphy, 1983). Our final objective was to characterize the Ca\(^{2+}\) dependence of the latch state in intact swine carotid media by decreasing the level of depolarization and monitoring stress maintenance as [Ca\(^{2+}\)] declined.

**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 at 22°C, as described by Driska et al. (1981). The intima was not removed in three experiments, although the endothelial lining was virtually absent, as assessed by light microscopy PSS consisted of (mm): NaCl, 140, KCl, 5; 3-[N-morpholino]propanesulfonic acid (MOPS, pH 7.4, at 22°C), 2; CaCl\(_2\), 1.6; MgCl\(_2\), 1.2; d-glucose, 5.6. Stimulating solutions were 109 mm KCl (KCl substituted stoichiometrically for NaCl in PSS) that was added by draining the organ bath by a vacuum line and then adding K\(^{++}\)-PSS Preparations that did not produce 0.5 x 10\(^{-9}\) N/m\(^2\) on depolarization at 22°C or develop 50% of the final stress after 2 minutes of depolarization were discarded.

Aequorin (obtained from Dr John Blinks, Mayo Medical School, Rochester) was loaded by a modification of the method of Morgan and Morgan (1982, 1984). Arterial strips were incubated successively at 2°C in the following solutions (mm): (1) 30 minutes in ethyleneglycol bis(β-aminoethyl ether)N,N,N\(^{\prime}\),N\(^{\prime}\)-tetraacetic acid (EGTA), 10; KCl, 120; adenosine 5'-triphosphate (ATP), 5; MgCl\(_2\), 2; N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES), 20 (pH 6.8 at 2°C); (2) 90 minutes in aequorin, 0.01; EGTA, 0.1; KCl, 120; ATP, 5; MgCl\(_2\), 2; TES, 20 (pH 6.8 at 2°C); (3) 30 minutes in EGTA, 0.1; KCl, 120; ATP, 5; MgCl\(_2\), 10; TES, 20 (pH 6.8 at 2°C); and (4) 30 minutes in NaCl, 140; KCl, 5; MgCl\(_2\), 10; MOPS, 2 (pH 7.1 at 22°C). This last solution was gradually warmed to 22°C, and CaCl\(_2\) was added to reach the following concentrations at 10-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 at 22°C. The penicillin and streptomycin were washed out before experiments were begun.

**Light Detection**

Light measurements were made in a light-tight enclosure modeled on the apparatus of Morgan and Morgan (1984) and Blinks (1982). The arterial strip was in a water-jacketed organ bath that has a circulating perfusion system driven by bubbling air through a side port. Room air was used, because 100% oxygen occasionally caused increased basal light and tone. Perfusion fluids could be replaced by a suction line and added by a second line. To rule out solution change artifacts, we monitored the light signal both during an exchange of PSS and during stimulation. Preparations which exhibited more than a 10% change during a PSS exchange were discarded. The arterial strip was placed at the focus of one ellipsoidal mirror (Melles Griot). An EMI 9635QA photomultiplier tube (Thorn EMI), selected for high gain and low noise, was placed at the focus of a second ellipsoidal mirror. A Bakelite shutter could be imposed between the two mirrors for visualization and manipulation of the strip without exposing the photomultiplier tube to ambient light. This prevents a persisting increased dark current (noise). The dark current of the photomultiplier tube estimated with the shutter closed was defined as zero. This was increased 0.04 ± 0.08 nA 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 0.2–0.5 nA) reflects the light emission by aequorin in unstimulated preparations. The photomultiplier output was amplified and filtered (usually with a 100-msec time constant) by a circuit of our design. Force was measured by a Grass FT.03 bridge transducer. The compliance of the system was 4.2 mm/kg. Force was displayed on a rectilinear recorder (Linseis model 2045) in synchronisation with the light signal.

**Calibration**

At the end of each experiment, 5 mm CaCl\(_2\) in water replaced the PSS to lyse the cells. This procedure completely lysed the tissue, as addition of 0.5% Triton X-100 did not result in additional light production. The large light signal was recorded until all the aequorin was discharged. The area under the curve was measured as nA-sec. \(L_{\text{max}}\), the peak light produced by injecting a similar amount of aequorin into a saturated CaCl\(_2\) solution, was calculated by the method of Allen and Blinks (1978). This procedure assumes there is a temperature-dependent time constant that describes the exponential decay of light after aequorin instantaneously contacts saturated Ca\(^{2+}\). Hastings (1969) reported the time constant to be 0.8 second at 20°C. Thus, the peak light, \(L_{\text{max}} = \) area (nA-sec)/0.8 sec. The exact value of the time constant is not critical because the same estimate was used to calculate \(L_{\text{max}}\) during calibration of both Ca\(^{2+}\)/EGTA solutions (see below) and tissue Ca\(^{2+}\). As \(L_{\text{max}}\) is a measure of the total undischarged aequorin present in the tissue, the ratio of light: \(L_{\text{max}}\) should be invariant with respect to the efficiency of loading in different tissues. In experiments at 37°C, the area under the light curve was calculated both during and at the end of each experiment because consumption of aequorin was significant. \(L_{\text{max}}\) was calculated individually for each time point by including the area under the curve of all subsequent light production. A "time constant" of 0.4 second (D.G. Allen, personal communication) was used to calculate \(L_{\text{max}}\) at 37°C.

Aequorin was calibrated by injecting 10 μl of 50 mm aequorin with a Hamilton 20-μl constant-rate syringe into
Comparison of stress produced and myosin light chain phosphorylation. Phosphorylations were determined on both unloaded strips immediately after finding L, and on “sham-loaded” strips that underwent the MgCl₂ loading L, and on “sham-loaded” strips that underwent the MgCl₂ loading. Stress was measured isometrically with a Grass FT.03 force transducer. For control experiments (Fig. 1), stress was calculated as percent of the KCl-induced stress initially obtained after determination of Lₚ prior to the loading procedure. Thus, each strip served as its own control.

Shortening velocity at zero load was estimated using isotonic quick-releases to varied afterloads (Dillon et al., 1981). A Cambridge Technology dual mode servo (model 300H) with a lever (model 350) was interfaced to a Northstar Horizon microprocessor. Software designed by Peter Becker (in preparation) allowed storage of results of four to five releases to fractional loads and calculation of isotonic velocity at those loads using an exponential curve-fitting program of tissue length at 200 time points from 1 to 2 seconds after release. Vₑ was extrapolated by the method of least squares, using a linearized Hill plot of the ratios Fₑ/Fₒ to (1 - Fₑ/Fₒ)/velocity when Fₒ was the load on release and Fₑ was the isometric force at the time of the release. Vₑ is the reciprocal of the y intercept.

Viability Tests of Aequorin Loading

In aequorin-loaded and myosin phosphorylation preparations, stress was measured isometrically with a Grass FT.03 force transducer. For control experiments (Fig. 1), stress was calculated as percent of the KCl-induced stress initially obtained after determination of Lₚ prior to the loading procedure. Thus, each strip served as its own control.

Shortening velocity at zero load was estimated using isotonic quick-releases to varied afterloads (Dillon et al., 1981). A Cambridge Technology dual mode servo (model 300H) with a lever (model 350) was interfaced to a Northstar Horizon microprocessor. Software designed by Peter Becker (in preparation) allowed storage of results of four to five releases to fractional loads and calculation of isotonic velocity at those loads using an exponential curve-fitting program of tissue length at 200 time points from 1 to 2 seconds after release. Vₑ was extrapolated by the method of least squares, using a linearized Hill plot of the ratios Fₑ/Fₒ to (1 - Fₑ/Fₒ)/velocity when Fₒ was the load on release and Fₑ was the isometric force at the time of the release. Vₑ is the reciprocal of the y intercept.

Microscopy

Both aequorin-loaded and control medial strips were fixed in 1.5% glutaraldehyde at 22°C for 2 hours, post-fixed with osmium tetroxide, and examined by transmission electron microscopy.

Statistics

Regression lines were calculated by the method of least squares. Forces were compared by Student's unpaired t-test. P values < 0.05 were considered significant. All results are expressed as means ± 1 SEM.

Calibration Procedures and Light Signals

Light production by aequorin is proportional to [Ca⁺²]³ and is quantified using the ratio L/Lₚ, where L is the light emission and Lₚ is a normalization for the tissue active aequorin concentration (Allen and Blinks, 1979). Note that L and Lₚ do not refer to tissue length. Aequorin light signals are reported as the logarithm of L/Lₚ, and this is proportional to the logarithm of [Ca⁺²] in the immediate vicinity of the aequorin. A calibration curve for light emission by aequorin where Ca⁺² is determined by a calcium-EGTA buffer system is shown in Figure 2. MgCl₂ competes with Ca⁺² and shifts the curve to the right. We also measured light production at a MgCl₂ of 0.5 mM, which is near the [MgCl₂⁺], of 0.4 mM estimated by nuclear magnetic resonance.
Figure 2. Calibration curve of log Lmax produced vs Ca** for Mg** concentrations of 0 (open circles), 0.5 mM (closed circles), and 1.25 mM (asterisks). The curves shown for 0 and 1.25 mM Mg** were provided by Dr. John Blinks with this batch of aequorin. The measured points and other curves were produced in this laboratory. Conditions were (mM): KCl, 120; TES, 20; EGTA, 1; pH 7.1; at 22°C. Also shown in squares and a dotted regression curve is the calibration of Ca**-buffered activating solutions used for determining the Ca** dependencies of stress and phosphorylation in skinned carotid media preparations.

in swine carotid media (Dillon, 1985). The calibration curve was linear from 0.3 to 10 μM Ca** at 0.5 mM Mg**, with a linear regression of pCa = 0.382 log Lmax - 4.40 (r = 0.9997). This calibration was used for calculation of [Ca**] in Figures 4, 5, and 7. We also calibrated aequorin light production in the contracting solutions used with "skinned" carotid media tissues (Chatterjee and Murphy, 1983) containing varying [Ca**] set by an EGTA/ATP buffer system used in our laboratory. Mg** was 0.94–0.96 mM in these solutions. These calibrations were used to compare data from skinned fibers with aequorin-loaded intact arterial preparations.

The light signals (Figs. 3 and 6) are quantified in both L:Lmax and nA. L:Lmax corrects for variations among tissues in the average amount of active aequorin present intracellularly. As Lmax was determined at a lower osmolality than that during contraction, we measured Lmax for aequorin in 5 mM CaCl2 at 22°C (hypoosmotic solution: Lmax = 1836 ± 146 μA/pmol aequorin). This is comparable to the value obtained in PSS (normo-osmotic solution: Lmax = 1922 ± 175 μA/pmol aequorin). These mean Lmax values were statistically similar (t = 0.39, n = 14), so Lmax was considered to be independent of ionic strength for calculation.

The resting light signal was high after loading strips with aequorin. Adding calcium back to the solution bathing the tissues increased the light signal with each increment. After a CaCl2 of 1.6 mM was reached, the light signal decayed slowly with a time constant of 30–90 minutes. This suggests that a pool of aequorin was discharged in which the average calcium concentration was approximately 1.4 μM (assuming [Mg**], to be 0.5 mM) or 1.8 μM (if [Mg**] = 1.25 mM). These [Ca**] are inferred on the basis of an aequorin consumption half-life of 1 hour, which predicts a log L:Lmax of −3.8 (see Appendix for derivation of L:Lmax from consumption half-life data). After approximately 16 hours at 22°C, this pool was decreased by 16 half-lives (approximately 65,000-fold), and basal light production was effectively stable. Approximately 99% of the active aequorin present in the tissue was consumed during this equilibration period. Only those experiments in which basal log L:Lmax was less than −4.75 were accepted and included in calculations (see below for discussion of this criterion). Some 25% of the experiments met this and the mechanical criteria. Our average basal L:Lmax of −4.92 ± 0.12 predicted a basal [Ca**] of 0.53 μM ([Mg**] = 0.5 mM) or 0.64 μM ([Mg**] = 1.25 mM) and an aequorin consumption half-life of 13.3 hours. Thus, waiting 16 hours (14–20 hours in various experiments) decreased the intracellular pool by only half. This waiting period selects for aequorin preferentially held in pools with [Ca**] in the <0.5 μM range.

At 37°C, the "time constant" of aequorin light decay was decreased and the consumption of aequorin was doubled. During contractions, the basal light output decreased significantly with time. However, when light was corrected for decreases in aequorin concentration (L:Lmax), the ratio L:Lmax in resting tissues was invariant for each preparation. This is to be expected if the resting [Ca**] remained constant over the period of experimental measurements. We measured basal light, Lmax, and the ratio L:Lmax at the beginning (Fig. 3B, label C) and end (Fig. 3B, label D) of nine experiments at 37°C. By the end of these experiments, the basal light signal fell 43 ± 7% (SEM) and Lmax fell 37 ± 6%. However, the ratio L:Lmax fell only 10 ± 5% (P < 0.01 for L:Lmax vs. either light or Lmax).

The active intracellular aequorin concentration at the beginning of the first contraction (label C in Fig. 3B) was estimated by dividing Lmax by both the Lmax of a known concentration of aequorin and its cell volume (estimated by weight, assuming a density of 1.05 g/ml and a previously determined cell fraction [60% (Murphy et al.,
1974). At 22°C with [Mg$^{++}$] = 0.5 mM, this batch of aequorin produced 1,922 μA/pmol aequorin. In five preparations (from Fig. 4), the mean estimate for the cellular concentration of active aequorin was 7.5 ± 3.3 pm. This predicts a loading efficiency, defined as the intracellular concentration of active aequorin divided by the concentration of aequorin in loading solution no. 2, of 0.00008%.

At 37°C, the quantum yield of aequorin was 79% of the yield at 22°C. However, due to the lower time constant at this temperature, $L_{max}$ was 3,030 μA/pmol aequorin. In six preparations (from Fig. 5), the mean active aequorin concentration was 5.0 ± 1.2 pm, with a loading efficiency of 0.00005%. The Ca$^{++}$-binding capacity of active aequorin in these preparations is 50,000 times less than that of quin 2 (at a concentration of 1 mM, a typical concentration used to estimate cell Ca$^{++}$). If inactive aequorin were to bind significant amounts of Ca$^{++}$, its concentration would need to be at least four orders of magnitude greater than the concentration of active aequorin (Blinks et al., 1978). The estimated concentration of aequorin was much lower than that reported by Fabiato (1985) in skinned myocardial cells. This poor loading efficiency probably reflects the low membrane permeability for a 20,000 dalton protein during the loading procedure. No estimate of total (active plus discharged) aequorin concentration was made. Johnson et al. (1985) reported a total aequorin concentration of 10 nm in platelets loaded with $^{125}$I-labeled aequorin, using a similar technique (a loading efficiency of 0.1% total aequorin). The aequorin supplied by John Blinks is typically 90% inactive, so the maximal intracellular active aequorin concentration in platelets would be 1 nm. This is 100–200 times the concentration observed in swine carotid. However, Johnson et al. (1985) did not use a 16-hour equilibration period, during which we observed approximately 99% aequorin consumption. This may explain our lower loading efficiencies.

**Representative Light Signals**

A representative light signal after depolarization by 109 mM KCl at 22°C is shown in Figure 3A. Light gradually rose to a peak in 3–4 minutes, and very slowly decreased, remaining significantly above baseline at 30 minutes. Stress rose with a similar time course and was maintained at peak levels.

Light signals after stimulation with KCl at 37°C showed two separate patterns, depending on how the solutions were changed. The method used most often to change solutions involved draining the bath and adding 50 ml of solution. This procedure required about 90 seconds through the lengthy tubing connections into the light-tight apparatus. Approximately 4 ml of PSS remained in the organ bath around the muscle after draining. This prevents artificial light signals due only to the solution change. A representative light signal for these con-
Correlations among Light, Phosphorylation, and Mechanical Measurements

Mean values of L:L\textsubscript{max}, phosphorylation, and V\textsubscript{o}, all rose to peak levels at 3–5 minutes, and then fell slightly after KCl depolarization at 22°C (Fig. 4). Developed stress was maintained for the 30-minute period. The transient in phosphorylation or V\textsubscript{o} upon depolarization was much smaller at 22°C (Fig. 4) than at 37°C (Fig. 5). The highest values of L:L\textsubscript{max}, phosphorylation, shortening velocity, and stress at 37°C were measured at 60 seconds (Fig. 5). Stress was maintained over the next 30 minutes while light fell approximately 28%, phosphorylation fell by 52%, and shortening velocity decreased by 40%. The change in phosphorylation with KCl depolarization was greater at 37°C, primarily because of higher resting phosphorylation levels at 22°C (15 ± 1%) vs. 37°C (7 ± 1%). Tone in the unstimulated tissues was also elevated at 22°C.

Gerthoffer et al. (1984) reported that approximately 70% of maximal stress was maintained for at least 1 hour, while phosphorylation fell to virtually basal values by changing to 20 mM KCl after several minutes in 109 mM KCl. The initial total depolarization with 109 mM KCl was necessary, as a 30-minute exposure to 20 mM KCl (without prior 109 mM KCl contracture) attained a stress of only 42 ± 7% (n = 6) of that produced when a 109 mM KCl contracture preceded the 20 mM KCl. This protocol produced a latch state with minimal myosin phos-
phorylation (Figs. 6 and 7). L:L_max fell dramatically with the reduction in [K⁺], despite maintenance of most of the developed stress. Upon return to PSS, L:L_max immediately fell to the baseline, while stress decayed slowly. Figure 7 shows mean data for all four measurements. At 30 minutes, light had fallen to 17% of the peak value, phosphorylation to 9%, velocity to 19%, while stress maintained at 65% of peak stress. Phosphorylation levels at 35 minutes were not significantly different from unstimulated tissues, although artifacts in accurately determining very low phosphorylation values (Aksoy et al., 1983) would prevent detection of small elevations.

Discussion

Evaluation of the Method

The technique used to measure [Ca++] is based on a procedure which Morgan and Morgan (1982, 1984) developed and applied to a variety of smooth muscle preparations. These workers conservatively presented most of their results as photomultiplier records of light emission correlated with changes in contractile force (Morgan and Morgan, 1982, 1984; DeFeo and Morgan, in press), as there was no method to verify calculated estimates of [Ca++]. The swine carotid media offers advantages for assessing the method. (1) The carotid media contains 60% by volume smooth muscle cells and less than 1% other cell types which could affect the light signal (Murphy et al., 1974). (2) The preparation is mechanically well characterized in terms of cellular output (Driska et al., 1978; Murphy, 1980). (3) Detailed correlation between myosin phosphorylation and mechanical activation are available (Dillon et al., 1981; Aksoy et al., 1983), and these have been related to [Ca++] in skinned medial tissues (Chatterjee and Murphy, 1983). The Ca++-stimulated phosphorylation and mechanical responses of these skinned tissues are comparable to intact preparations.

Critique of the Aequorin-Loading Procedure

The lengthy loading procedure did not adversely affect the mechanical properties or myosin light chain phosphorylation in most preparations. However, approximately 75% of the loaded muscles failed to meet the criteria that the basal light signal was stable for at least 30 minutes and had a log L:L_max less than -4.75. If [Mg++] is assumed to be 0.5 mM (Dillon, 1985), the average basal light production of the 37°C preparations (log L:L_max = -4.92 ± 0.12) predicted a basal [Ca++] of 0.53 µM. If a [Mg++] is assumed to be 1.25 mM, basal [Ca++] was 0.64 µM.

The increased basal light production of the excluded preparations could be due to many factors. (1) Increased average permeability of the plasma membrane to Ca++ could elevate the [Ca++], throughout the preparation. This should cause increased basal tone, and this was observed in a small number of cases.
number of these preparations with high resting light levels. Similar elevations in tone were seen in some unloaded carotid media preparations after dissection and 1 day in 22°C PSS. (2) A subpopulation of cells damaged by dissection, the loading procedure, or in vitro conditions could account for increased light without increased resting tone. If 1% of the cells were dying and had a [Ca++] of 3.0 μM, the resting light signal would approximately double. This possibility is consistent with much experimental work on in vitro smooth and striated muscles, in which an equilibrium period after dissection is necessary to allow for cell recovery or death leading to reproducible responses. The exponential light decay during the lengthy equilibration period after loading may reflect a mixture of cell recovery and death which continues at low rates in vitro. (3) Aequorin may slowly leak or be transported into the extracellular space, where it contacts millimolar Ca++ and increases resting light emission. (4) Aequorin could be loaded into another pool (e.g., sarcoplasmic reticulum) that has a higher [Ca++]]. However, any aequorin in such compartments would be preferentially consumed. Thus, only pools with resting [Ca++] less than 0.5 μM should have significant levels of active aequorin after 16 hours. Mitochondria have been reported to have the same total calcium as the cytoplasm (Somlyo, 1985). If the free Ca++ is similar, the mitochondria may contribute to the basal light signal, but would not produce a significant artifact in the estimation of [Ca++]]. (5) A final consideration is that [Ca++] in unstimulated preparations is close to the minimum concentrations at which Ca++ can be reliably measured with aequorin (Fig. 2). At 37°C, Ca++-independent luminescence occurs at a log L:Lmax near −5.8 (Blinks, 1982). This limit depends on both [Mg++] and temperature. The L:Lmax for unstimulated tissues is not the minimally detectable light emission, as subtitution of PSS with an EGTA-containing Ca-free PSS produced a fall in L:Lmax, in agreement with the results obtained by Morgan and Morgan (1984).

In summary, high basal light emission is a serious problem and necessitates a stringent criterion for analysis. Our limit of a log L:Lmax of −4.75 (with an average L:Lmax = −4.92 ± 0.12 at 37°C) is arbitrary, and we may overestimate resting [Ca++]p. The increment in light on stimulation was larger in those preparations with high resting light than in those with low resting light. Similar elevations in tone were seen in some preparations in light on stimulation was larger in those preparations with high resting light than in those with low resting light. The increase in L:Lmax, on stimulation, the error bars in average data (Fig. 5) decreased by 69 ± 3%. This analysis has two implications. First, K+-produced increases in aequorin light are highly reproducible, as are the mechanical responses. Second, variation in tissues accepted for analysis was due primarily to factors affecting the determination of Lmax. Difficulties in measuring Lmax may explain some of the variation in basal light production between different preparations. Lmax is calculated by measuring total light production and dividing by the decay constant for aequorin in millimolar Ca++ (Hastings et al., 1969). The Lmax of aequorin was independent of ionic strength, and so the use of a hypoosmotic calcium solution to rupture the cells and discharge aequorin should not affect the determination of Lmax.

Inhomogeneities in the distribution of aequorin or Ca++ can introduce large errors into the aequorin light signal calibration (Blinks et al., 1982a). During a typical 30-minute 109 mM KCl contraction at 37°C, approximately 40% of the active aequorin is consumed. Thus, the light signal cannot be from a small subpool of aequorin contacting high Ca+++, because a small pool would be rapidly consumed and the light signal would rapidly fall. Basal light production, when corrected for consumption (L:Lmax), is relatively unaffected by a 30-minute 109 mM contraction (fell 10 ± 5%, n = 5), despite the 40% aequorin consumption. This suggests that the active aequorin that is present is in one large pool. We cannot determine whether Ca++ inhomogeneities exist; however, by emphasizing relatively steady state comparisons, we can minimize diffusionally dependent Ca++ gradients.

Active aequorin may not be uniformly loaded, and detection of photon emission may have different efficiencies in different tissue layers. Although aequorin is a large molecule, diffusional calculations showed that equilibration should occur in the interstitial space at the center of the tissue during the 90-minute loading period. Radiolabeled aequorin could address the uniformity of total aequorin loading, but it would not demonstrate uniformity in the small fraction of aequorin that remains active. If the relationship between [Ca++] and myosin phosphorylation is similar in aequorin-loaded tissues and other preparations (such as skinned fibers), this implies that inhomogeneities in aequorin loading or light detection are not grossly compromising the results. Attenuation of the light signal from the interior of the tissue may occur. If we assume that the light attenuation of the tissue is not affected by cell lysis, similar attenuation would occur during determination of both L and Lmax. Thus, the ratio L:Lmax factors out the attenuation, even though this prejudices the signal toward the surface of the tissue.

Critique of Light Signal Calibrations

The second stage of the aequorin calibration is conversion of L:Lmax into [Ca++]]. The calibration is
dependent of myoplasmic \([\text{Mg}^{++}]\), ionic strength, and temperature (Blinks et al., 1982). Our attempt to approximate intracellular conditions with respect to those parameters known to affect light production is shown in Figure 2. The resting \([\text{Mg}^{++}]\), in vascular smooth muscle is controversial. Bond et al. (1984) measured total myoplasmic magnesium and suggested that \([\text{Mg}^{++}]\) may be appreciably greater than the 0.5 \(\mu\text{M}\) value estimated by nuclear magnetic resonance measurements in this tissue (Dillon, 1985). Since the results are given as log \(L: L_{\text{max}}\), \([\text{Ca}^{++}]\) estimates can be calculated readily if different \([\text{Mg}^{++}]\) values are assumed. Changes in \([\text{Mg}^{++}]\), during contraction are possible, and could affect the light signal. A 20% decrease in total magnesium concentration was reported in a KCl plus norepinephrine-stimulated contraction by Bond et al. (1984), who used electron probe analysis. However, Dillon (1985) reported that \([\text{Mg}^{++}]\), did not change during depolarization-induced contraction in the swine carotid. Due to limitations of our equipment, calibrations were obtained only at 22°C. Increasing temperature to 37°C will shift the calibration curve to the left by 0.1–0.2 log units (Blinks et al., 1982). Thus, \([\text{Ca}^{++}]\), at 37°C may be slightly less than that shown in Figures 5 and 7. Due to the steepness of the aequorin calibration curve and the uncertainties of intracellular conditions, values of \(L: L_{\text{max}}\) should provide accurate reflections of changes in \([\text{Ca}^{++}]\), but the absolute values of \([\text{Ca}^{++}]\), are subject to uncertainties and must be evaluated in conjunction with independent estimates.

We cannot show directly that the aequorin signals reflect pennymofibrillar \([\text{Ca}^{++}]\). However, we can rule out contributions from other locations in this tissue. (1) The 16-hour equilibration in PSS ensures complete consumption of any aequorin in the interstitial space or adsorbed to the glycothecy. (2) Rupture of cell membranes with hypooncotic solutions produces transient light levels five orders of magnitude larger than resting light levels. There were no light or mechanical responses to depolarization after cell lysis. All aequorin was in a location releasable by disruption of cell membranes. (3) Predicted basal \([\text{Ca}^{++}]\), levels in unstimulated preparations were slightly higher than measurements in many cell types using fluorescent dyes and microelectrodes where basal \([\text{Ca}^{++}]\), levels were found to be 0.05 to 0.3 \(\mu\text{M}\) (Rasmussen, 1981; Tsien, 1983; Yamaguchi, 1985). However, unstimulated preparations exhibit tone, particularly at 22°C. This implies that \([\text{Ca}^{++}]\), is elevated above levels required for complete relaxation. (4) The signals are similar to those reported by Morgan and Neering (1980), in their study in which individual cells were pressure-injected with aequorin. (5) Aequorin is rapidly discharged in pools such as sarcoplasmic reticulum that have a \([\text{Ca}^{+}+]\) greater than the myoplasm. The only remaining known locations for aequorin are the myoplasmic space, mitochondria, and nucleus. The volume of the latter two are a small fraction of the myoplasmic space. Fabiato (1985) reported that skinned cardiac Purkinje myofibrils concentrate aequorin. If a similar process occurs in smooth muscle, an aequorin signal due mostly to myofibrillar \([\text{Ca}^{++}]\) would be favored.

In summary, our “steady state” \([\text{Ca}^{++}]\), estimates (defined as 90-second after completion of a solution change) are in reasonable agreement with those obtained by different methods in a variety of tissues. However, estimates of resting \([\text{Ca}^{++}]\), levels are near the useful limit for this method, and there are uncertainties about the calibration of \(L: L_{\text{max}}\) in terms of \([\text{Ca}^{++}]\). The strongest argument that \(L: L_{\text{max}}\) reflects \([\text{Ca}^{++}]\), comes from correlations with \([\text{Ca}^{++}]\)-dependent myofibrillar responses (next section). For our primary objective, these changes in \(L: L_{\text{max}}\) are more important than derived calculations of \([\text{Ca}^{++}]\).

**Evaluation of the Results**

**Correlation of Steady State Light Emission with Phosphorylation and \(V_o\)**

Biochemical experiments established that myosin phosphorylation is \([\text{Ca}^{++}]\)-dependent (Kamm and Stull, 1985a), and data from intact tissues suggest that shortening velocity at zero load \((V_o)\) is directly proportional to myosin phosphorylation when measured during relatively steady state conditions (Aksoy et al., 1982; Weisbrodt and Murphy, 1985; Kamm and Stull, 1985b). Figure 8 shows the correlation of light emission (log \(L: L_{\text{max}}\)) with both myosin phosphorylation and \(V_o\) at 37°C. Below the log \(L: L_{\text{max}}\) scale is a \([\text{Ca}^{++}]\) calibration assuming \([\text{Mg}^{++}]\), = 0.5 \(\mu\text{M}\). This calibration is based on many assumptions, and an independent test of its accuracy will be discussed below. Only data obtained in a relatively steady state are presented (see above). There was good correlation between log \(L: L_{\text{max}}\) and phosphorylation \((r = 0.97, n = 6)\) and between log \(L: L_{\text{max}}\) and \(V_o\) \((r = 0.995, n = 5)\). This supports the hypothesis that changes in myoplasmic \([\text{Ca}^{++}]\), are the determinants of myosin phosphorylation and crossbridge cycling in depolarized swine carotid media. This correlation is consistent with the fourth criterion of Krebs and Beavo (1979), as reviewed by Kamm and Stull (1985a) for acceptance of \([\text{Ca}^{++}]\)-stimulated myosin phosphorylation as a physiologically significant regulatory mechanism.

**\([\text{Ca}^{++}]\) Sensitivity of Myosin Phosphorylation and the Latch State**

The latch hypothesis was developed to explain stress maintenance with decreasing myosin phosphorylation and \(V_o\) in tonically depolarized swine carotid. As stress maintenance was \([\text{Ca}^{++}]\)-dependent, a second \([\text{Ca}^{++}]\)-dependent regulatory mechanism was hypothesized (Aksoy et al., 1983; Gerthoffer and Murphy, 1983; Chatterjee and Murphy, 1983). The protocol in Figure 7 produced large decreases.
in myosin phosphorylation despite stress maintenance and allowed estimation of the \( K_{50} \) levels for stress maintenance and phosphorylation. \( K_{50} \) is defined as the \([Ca^{++}]i\) for half-maximal stress or phosphorylation. Figure 9 shows the dependence of phosphorylation and stress on \( \log \frac{L}{L_{\text{max}}} \) and the calculated \([Ca^{++}]i\). These curves were generated by plotting successive time points of \( \log \frac{L}{L_{\text{max}}} \) vs. phosphorylation or stress from Figures 5 and 7. No time points less than 90 seconds after changing the KCl concentration were included, to avoid diffusion-limited errors. This differs from the usual dose-response method of calculating \( K_{50} \) levels because the \([Ca^{++}]i\) cannot be varied at will in intact tissue preparations. We can only measure changes in light resulting from changes in stimulation. Thus, estimates of \( Ca^{++} \) sensitivity must be interpreted cautiously. \( K_{50} \) values were estimated by fitting these points to the equation normalized stress (or phosphorylation) = \( \frac{1}{1+(K_{50}/[Ca^{++}]i)^{n}} \), as was done on the skinned fiber data by Chatterjee and Murphy (1983). For phosphorylation, the \( K_{50} \) was -4.20 (log \( \frac{L}{L_{\text{max}}} \)), which predicted a \([Ca^{++}]i\) of 0.99 mM (\([Mg^{++}]i\) = 0.5 mM) or 1.25 mM (\([Mg^{++}]i\) = 1.25 mM, \( N = 6.7; P < 0.0005 \)). For stress, the \( K_{50} \) was -4.89 (log \( \frac{L}{L_{\text{max}}} \)), which predicted a \([Ca^{++}]i\) of 0.54 mM (\([Mg^{++}]i\) = 0.5 mM) or 0.65 mM (\([Mg^{++}]i\) = 1.25 mM, \( N = 6.9; P < 0.000001 \)). As discussed above, aequorin measurements near the basal light level are subject to a number of errors, all of which tend to overestimate \([Ca^{++}]i\). Thus, the \( K_{50} \) for stress is more of an upper bound than an accurate measure of sensitivity. This method is not applicable for estimation of the \( K_{50} \) for stress development due to the slow kinetics of solution changes discussed above. This analysis shows that the \( Ca^{++} \) sensitivity of stress maintenance is greater than that of myosin phosphorylation.

Comparison of Estimated \([Ca^{++}]i\), with Biochemical and Skinned Fiber Data

Due to the potential inaccuracies in the calibration of aequorin, we sought to check independently the
Ca++-sensitivity estimates, using both biochemical and skinned fiber data. Using an empirically derived equation (Blumenthal and Stull, 1980) or a multi-equilibrium model (Rembold et al., 1985), we estimated that K50 values for Ca++-dependent MLCK activation were 0.98 and 0.94 μM, respectively. These estimates are based on available data for the calmodulin concentration (20 μM) and empirically derived binding constants in vascular smooth muscle (Rembold et al., 1985). The K50 for MLCK activity is not necessarily equivalent to the K50 for myosin phosphorylation, as the latter is also dependent on phosphatase activity and calmodulin binding to other proteins. However, this analysis does give a general range for expected levels of [Ca++] based on purely biochemical data. Unfortunately, the biochemical basis of stress maintenance has yet to be elucidated, so comparison is not possible.

Skinned preparations allow direct determination of the relationship between [Ca++], and myosin phosphorylation. Table 1 compares the aequorin-estimated Ca++ sensitivity for phosphorylation and stress with skinned fiber data. Chatterjee and Murphy (1983) reported the Ca++ dependence of initial stress development and myosin phosphorylation when levels of [Ca++] were increased, and the Ca++ dependence for stress maintenance and myosin phosphorylation when preparations contracted in high [Ca++] were exposed to lower levels of [Ca++].

The K50 for myosin phosphorylation was independent of the starting [Ca++], and the K50 for initial stress development was similar to the phosphorylation K50. However, the K50 for stress maintenance was nearly a log unit lower. Aequorin estimates in intact tissues (Fig 9) are equivalent to the decreasing Ca++ protocol in skinned fibers. The Ca++ dependencies for myosin phosphorylation in intact and skinned preparations are comparable (Table 1). The aequorin-derived K50 estimates for stress maintenance are significantly lower than for myosin phosphorylation. They are, however, somewhat higher than the value measured in skinned tissues. This may be explained by the limitations of Ca++ calibration when the light emission level is close to basal light production as described above. This comparison with skinned tissues provides an independent test of the Ca++ calibration. The aequorin method provides fairly reasonable estimates of [Ca++], for nontransient conditions during depolarization. Whereas uncertainties about absolute values of [Ca++], remain, the relative Ca++ dependence of phosphorylation, V0, and stress can be determined in intact tissues.

Conclusions

(1) Aequorin-estimated myoplasmic Ca++ is proportional to myosin phosphorylation and mean crossbridge cycling rates in depolarized swine carotid media. (2) In intact tissues, stress can be maintained at levels of [Ca++], which do not support proportional phosphorylation. This difference in Ca++ sensitivity would allow vascular smooth muscle to control both rapid stress development (with phosphorylated crossbridges) and stress maintenance (with dephosphorylated, non-, or slowly cycling crossbridges—"latch") using a single second messenger.

Appendix

Derivation of the Consumption Half-Life of Aequorin from L(t)

Consumption appears to be exponential (Blinks et al., 1982). Thus:

\[ L = L_0 e^{-0.693 t/t_{1/2}} \]

where \( L_0 \) is light at \( t = 0 \), and \( t_{1/2} \) is the half-life of aequorin consumption at a specific [Ca++] as Lmax = area in nA·seconds / 0.8 second at 22°C, the.
Thus:

\[ L_{\text{max}} = \frac{1}{0.8} \int_0^\infty \text{L}_0 e^{-0.69t/t_{1/2}} \, dt \]

By dividing \( L \) by \( L_{\text{max}} \), setting \( t = 0 \), and evaluating the integral:

\[ L/L_{\text{max}} = (0.8)(0.69)/t_{1/2} \]

Thus:

\[ t_{1/2} = 0.552/(L:L_{\text{max}}) \]

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