Cytoplasmic Free Calcium, Myosin Light Chain Phosphorylation, and Force in Phasic and Tonic Smooth Muscle

BERNARD HIMPENS, GERT MATI'HIJS, AVRIL V. SOMLYO, THOMAS M. BUTLER, and ANDREW P. SOMLYO

From the Pennsylvania Muscle Institute, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104-6083; and the Thomas Jefferson University, Department of Physiology, Philadelphia, Pennsylvania 19107

ABSTRACT The time course of \([\text{Ca}^{2+}]_i\), tension, and myosin light chain phosphorylation were determined during prolonged depolarization with high \(K^+\) in intact tonic (rabbit pulmonary artery) and phasic (longitudinal layer of guinea pig ileum) smooth muscles. \([\text{Ca}^{2+}]_i\), was monitored with the 340 nm/380 nm signal ratio of the fluorescent indicator fura-2. The fluorescence ratio had a similar time course in both muscle types during depolarization with 109 mM \([K^+]_o\); after a transient peak, there was a decline to 70% of its peak value in tonic smooth muscle, and to 60% in phasic smooth muscle. Tension, however, continued to increase in the pulmonary artery, while in the ileum it declined in parallel with the \([\text{Ca}^{2+}]_i\). On changing \([K^+]_o\) from 109 to 20 mM, tension and \([\text{Ca}^{2+}]_i\), either remained unchanged or declined in parallel in the pulmonary artery. Phosphorylation of the 20-kD myosin light chain, measured during stimulation of muscle strips with 109 mM \([K^+]_o\) in another set of experiments, increased from 3% to a peak of 50% in the intact pulmonary artery, and then declined to a steady state value of 23%. In the intact ileum, a very rapid, early transient phosphorylation (up to 50%) at 2-3 s was seen. This transient declined by 30 s to a value that was close to the resting level (7%), while tension remained at 55% of its peak force. A quick release during maintained stimulation induced no detectable change in the \([\text{Ca}^{2+}]_i\), in either type of smooth muscle. We discuss the possibility that the slowly rising tonic tension in pulmonary artery could be due to cooperativity between phosphorylated and non-phosphorylated crossbridges.

INTRODUCTION

Activation of vertebrate smooth muscle is triggered by a rise in cytoplasmic free \(\text{Ca}^{2+}\) (reviewed in Somlyo, 1985) that combines with calmodulin, the catalytic subunit of myosin light chain kinase (MLCK), to form the active \(\text{Ca}^{2+}\)·calmodulin-MLCK complex. Contraction is thought to be initiated when phosphorylation of myosin light chains by MLCK permits the activation of myosin ATPase by actin.
(Kamm and Stull, 1985; Hartshorne, 1987). According to this model, relaxation is secondary to a fall in Ca\(^{2+}\) and a resultant decrease in MLCK activity, accompanied by dephosphorylation of myosin by light chain phosphatases (Haeberle et al., 1985; Hoar et al., 1985; Ruegg, 1986).

An important property, unexplained by the above simple model, is the ability of smooth muscle to maintain force over prolonged periods at low energy cost (Siegman et al., 1980) and at low levels of myosin light chain phosphorylation (Dillon et al., 1981; Silver and Stull, 1982). It is not known whether the decline in myosin light chain phosphorylation is due to a fall in cytoplasmic Ca\(^{2+}\) or whether force is maintained during this time by a Ca\(^{2+}\)-insensitive mechanism(s) or by a Ca\(^{2+}\)-sensitive mechanism other than myosin light chain phosphorylation/dephosphorylation. The existence of two types of smooth muscle, tonic and phasic (Somlyo and Somlyo, 1968, 1969; Somlyo et al., 1969), also raises the possibility that differences in contractile regulation may be type specific (Weisbrodt and Murphy, 1985). We have therefore used in this study fura-2, a fluorescent Ca\(^{2+}\) indicator (Grynkiewicz et al., 1985), to determine the relationships between [Ca\(^{2+}\)]\(_i\), force, and myosin light chain phosphorylation in intact tonic and phasic smooth muscles. In the course of these studies we also encountered an extremely rapid myosin light chain phosphorylation transient in intact ileal smooth muscle.

**Methods**

**Tissue Preparation**

Male and female New Zealand white rabbits, weighing ~3 kg were instantaneously killed by cervical dislocation by a humane procedure, as approved by the Institutional Animal Care and Use Committee. The heart and lungs were removed and transferred to a warm oxygenated Krebs solution. The left and the right pulmonary arteries were excised and cleaned of their periarterial connective tissue and the endothelium was gently scraped away. The pulmonary artery of the rabbit was used, because the properties of this tonic smooth muscle have been well characterized (Somlyo and Somlyo, 1968, 1969; Somlyo et al., 1969).

The longitudinal layer of guinea pig ileum was chosen as an example of phasic smooth muscle, because its behavior with different fluorescent indicators is well known. A description of its dissection can be found in Himpens and Casteels (1987) and Himpens and Somlyo (1988). Attempts to load the guinea pig and rabbit portal vein with fura-2AM were unsuccessful.

**Fura-2 Measurements**

The protocols for loading with fura-2AM and for making the fluorescence measurements have been described by Himpens and Somlyo (1988). Briefly, the procedure consists of loading the strip in a cuvette containing 1 ml of HEPES-buffered Krebs solution containing 1.2 mM Ca\(^{2+}\) and 3 μM fura-2AM dissolved in dimethyl sulfoxide (final concentration, 0.5%) premixed with Pluronic F127 (final concentration, 0.01%). Strips were loaded at room temperature (with the cuvettes rotating at 30 rev/min) overnight for the pulmonary artery and for 4 h for the longitudinal ileum. We have previously measured the concentration of cytoplasmic free fura-2 achieved by our loading procedures in the ileum, and we have established that this had no effect on the rate or the amplitude of force development (Himpens and Somlyo, 1988). The strips, transferred from the loading solution to a fresh Krebs solution, were then washed for 1 1/2 h. The method of mounting and a description of the apparatus
have been published (Himpens and Somlyo, 1988). During the quick-release experiments muscle length was manually changed by ~10% in 1 s. The calculations used to estimate stress were based on strip lengths at $L_0$ and on the tissue wet weight. Stress was expressed in: $N/m^2 = (force\ in\ g) \times (9.807 \times 10^{-3} \ N/g) / (cross-sectional\ area\ in\ m^2)$. The cross-sectional area was determined as: (wet weight in kilograms)/(length at $L_0$ in meters) (1,050 kg/m$^2$) (Murphy, 1980).

Cytosolic $Ca^{2+}$ concentrations were calculated as described by Grynkiewicz et al. (1985) using the following formula:

$$[Ca^{2+}] = K_d \times \frac{S_n}{S_d} \times \left[\frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)}\right].$$

(1)

$K_d$, the apparent dissociation constant of the $Ca^{2+}$/fura-2 complex, was assumed to be 224 nM, as measured by Grynkiewicz et al. (1985). $R$ is the experimentally determined ratio of fluorescence emission (at 510 nm) at the two excitation wavelengths of 340 and 380 nm (corrected for the autofluorescence background at each wavelength). $R_{\text{max}}$ is the maximal corrected ratio measured in the presence of saturating $Ca^{2+}$, while $R_{\text{min}}$ is the ratio obtained with $Ca^{2+}$-free solution. The constant $S_n/S_d$, which corrects for the instrument conditions, path-length, etc., is the ratio of the background subtracted fluorescence intensities of the free dye/$Ca^{2+}$-bound dye at 380 nm.

Ionomycin (50 nM) was used to equilibrate intra- and extracellular $Ca^{2+}$. Only this (high) concentration of the ionophore induced contractions that were equivalent to maximal responses to agonist stimulation. Lower concentrations resulted in only partial or no contractions of the muscle. An internal calibration on an intact strip was preferred, because an external calibration, after lysis of the strip, could be disturbed by components of the medium or by the frequently observed lysis of cells, and because the dilution of the fura-2 from a strip in a tissue chamber can induce large volume errors in the calculation, particularly with the low intracellular fura-2 concentrations used in the experiments. Internal calibrations also minimized contributions of possible $Ca^{2+}$-insensitive metabolites of fura-2 (Scanlon et al., 1987).

The minimum fluorescence was obtained by superfusing the muscle with a 140 mM K$^+$, Ca$^{2+}$-free solution containing 2 mM EGTA at pH 8.6 to optimize the ionomycin effect (Liu and Hermann, 1978). 5 min after superfusion with this solution, 50 nM ionomycin was added. After determining the $R_{\text{min}}$, the tissue was superfused with an excess of calcium (10 mM Ca$^{2+}$ solution at pH 8.6), which gave the maximal signal ratio, $R_{\text{max}}$ (Fig. 1). After reaching a maximum, the fluorescence frequently declined, due to leakage of fura-2 and its subsequent removal from the superfused strip.

The autofluorescence was determined after each experiment at the two excitation wavelengths in order to subtract the values from the total fluorescence and to obtain the net fura-2 fluorescence. This was done by superfusing the strip with a 20 mM Mn$^{2+}$ Krebs solution after the $R_{\text{max}}$ and $R_{\text{min}}$ ratios were determined. Mn$^{2+}$ binds to fura-2 with a higher affinity than Ca$^{2+}$ (48-fold) and its entry is also facilitated by ionomycin. Mn$^{2+}$ quenches the fura-2 fluorescence, but does not affect the cell autofluorescence, the fluorescence of the acetoxymethylester (Luckhoff, 1986) or of the partial hydrolysis products of fura-2AM (Scanlon et al., 1987). Therefore, the value at each individual wavelength represents the proportion that has to be subtracted from each signal before making the ratio signal.

The term $S_n/S_d$ was obtained simply from the quotient of fura-2 fluorescence (380-nm excitation wavelength) measured during the minimal and maximum fluorescence signals.

The above calibration was used in each of the experiments in which absolute values of $Ca^{2+}$ are indicated in the figures. Results of earlier experiments, performed without the complete calibration described, are only shown as changes in the fluorescence ratios.

After this study had been completed, Becker and Fay (1987) reported the appearance of a photobleached fluorescent derivative of fura-2, and suggested certain precautions for elimi-
nating the errors in quantitation that may be introduced by such a photobleached product. We note that photobleaching is more likely to occur at the high radiation levels required for obtaining fluorescent signals from single cells (Becker and Fay, 1987), and it was shown to be absent, within detection limits, in our earlier study that used this method of using fura-2 in whole smooth muscle strips (Himpens and Somlyo, 1988). Furthermore, several of the precautions recommended by Becker and Fay (1987) are already implemented in our method; these include low O₂ concentration (our solutions are not oxygenated), minimal exposure to light (the experiments are conducted in a darkroom illuminated by red light only), and reduction of the amount of dye in the extracellular fluid (extracellular dye is removed by continuous perfusion). Accordingly, we do not believe that photobleached products are a significant source of error for quantitation in our experiments, but emphasize that the use of Kᵅ values determined in cuvette calibrations (Grynkiewicz et al., 1985) may not reflect the actual disso-

Figure 1. Calibration procedure. After increasing pH to 8.6 in a Ca²⁺-free depolarizing solution, ionomycin was injected in the bath to a final concentration of 50 μM. This frequently induced transient increases in [Ca²⁺], and tension, which were followed by a decline of the two signals to their minimum levels. Subsequently, the maximum tension and fluorescence ratio was obtained by increasing the external [Ca²⁺] to 10 mM. Finally, 20 mM Mn²⁺ is added to quench the fura-2 signal and to obtain the background fluorescence. In the upper part of the figure the two individual (corrected) signals (510-nm emission, at (A) 340- and (B) 380-nm excitation) are shown in a strip of rabbit pulmonary artery. (C) The fluorescence ratio signal; note that the washout in Mn²⁺ is left out of the figure, since the noise level is high (the two signals are approaching zero). D shows the force.

The diffusion time of the solutions into the extracellular space of the preparations in the chamber was estimated by using fura-2 acid as a fluorescent tracer in the perfusate. Complete penetration of the dye into the strips, as determined by attainment of the maximum ratio signal, required 30 s for the ileum and 60 s for the pulmonary artery (Fig. 2).

The standard physiological solution was a HEPES-buffered modified Krebs solution at pH 7.3 containing (in millimolar): 135.5 Na⁺, 5.9 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, 143.8 Cl⁻, 11.6 HEPES, and 11.6 glucose. Solutions with increased [K⁺]₀ were obtained by replacing Na⁺ with an equivalent amount of K⁺. Fura-2AM was obtained from Molecular Probes, Inc.
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(Junction City, OR), pluronic F127 from BASF Wyandotte Corp. (Parsippany, NJ), and verapamil was from Sigma Chemical Co. (St. Louis, MO).

Myosin Phosphorylation Measurements

Circular strips of one of the two main branches of the rabbit pulmonary artery or strips of the guinea pig ileum were attached to a force transducer (Somlyo et al., 1981), stretched to physiological length, mounted in muscle baths at room temperature in Krebs solution, and equilibrated for 30 min. Some strips were frozen in relaxed conditions, while others were stimulated for the required intervals by rapid immersion in a bath of 140, 109, or 20 mM [K+]o. The muscle bath was quickly lowered and a beaker of supercooled Freon 22 (Somlyo et al., 1977) was shot up to the muscle. The moment of freezing was detected on the tension trace.

The methods for homogenization of muscles and two-dimensional gel electrophoresis were similar to those described by Butler et al. (1983). Briefly, the frozen strips were pulverized in the presence of 0.5 N perchloric acid and after thawing, centrifuged. The protein precipitate was suspended in a urea-containing solution (Butler et al., 1983). This was subjected to isoelectric focusing, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The

FIGURE 2. Rate of penetration (diffusion time) of the free fura-2 acid (2 μM) into the extracellular space of strips of the pulmonary artery, displayed on two different time bases. 60 s after changing the perfusate from normal Krebs solution (right trace) to one containing fura-2, a stable maximal fura-2 340/380 ratio signal was reached. The extracellular location of the fura-2 is demonstrated by its fast disappearance during washout (left trace). The autofluorescence was not subtracted before determining the ratio in this figure, due to the noise level obtained by dividing two near-zero values (340- and 380-nm signal) before the appearance of fura-2. Slab gels were stained with Coomassie Blue and, if necessary, were subsequently silver stained (Merril et al., 1981). Densitometry of the 20-kD region of the gel allowed quantitation of the fraction of the total myosin light chain that was phosphorylated.

In some muscles (n = 4) myosin phosphorylation was measured after loading with fura-2. Myosin phosphorylation during stimulation was not affected when compared with unloaded preparations (data not shown).

Statistical Analysis

All values are means ± SE, and n is the number of observations. Comparisons were made using Student's t test.

RESULTS

Tonic Smooth Muscle: Rabbit Pulmonary Artery

The resting [Ca2+]i was 106 ± 8 nM (20 measurements made on 15 strips). After depolarization with 109 mM K+ at room temperature (n = 11), the fluorescence
ratio rose within 1–2 min to a maximum peak (390 ± 34 nM), after which it decreased to a lower plateau level (289 ± 20 nM), where it remained as long as the stimulus was maintained (Fig. 3). The initial increase to maximum [Ca²⁺]ᵢ was not accompanied by the rapid force transient previously observed in phasic smooth muscle (Himpens and Somlyo, 1988).

Phosphorylation at rest was 3% of the total, and increased within 90 s to its maximum (50%), declining thereafter to a steady state value of 23% (n = 3) after 30 min (Fig. 4). Force rose with a slow time course and continued to increase while [Ca²⁺]ᵢ and phosphorylation were declining; force was only 48 ± 6% of its maximum amplitude at the moment the [Ca²⁺]ᵢ was maximal. Force development was not rate-limited by diffusion of K⁺; tension developed more slowly than the rate of entry of the perfusate monitored by fura-2–free acid (see also Fig. 2).

We also measured [Ca²⁺]ᵢ during the high force/low phosphorylation state observed in swine carotid artery smooth muscle after the perfusion changed from 109 to 20 mM [K⁺]₀ (Rembold and Murphy, 1986). However, to avoid diffusional delays due to unstirred layers and elastic lamellae, we applied the solutions for a longer period (15 min) than described by Rembold and Murphy. When, after a 15-min superfusion with 109 mM [K⁺]₀, the K⁺ concentration was reduced to 20 mM K⁺ for 20 min, two types of response occurred: in 18 muscle strips (illustrated by Fig. 5) both the force and the fluorescence ratio declined during perfusion with 20 mM K⁺. The fluorescence ratio, normalized to the value maintained in 109 mM K⁺, declined after a transient increase during the initial superfusion of 20 mM K⁺ to 83 ± 4% during a maintained application of 20 mM K⁺. Force, which also underwent a transient increase when the perfusate was changed from 109 to 20 mM K⁺, declined to 75 ± 3% of the maximum value in 109 mM K⁺. However, this and the level of [Ca²⁺]ᵢ were still 25% higher than the respective parameters after a 40-min exposure of the strip to 20 mM K⁺ without the previous 109 mM K⁺ stimulation.

In the other 22 strips (illustrated by Fig. 6) neither tension nor fluorescence declined when perfusion was changed from 109 to 20 mM K⁺. The data representing both types of results (n = 40) are summarized in Fig. 7. We have no direct
evidence concerning the mechanism of the two different types of response to lowering K⁺ from 109 to 20 mM, and can only suspect that this biological variability may be the result of different levels of repolarization (not measured in this study) or different amounts of nerves and neurotransmitters remaining at the adventitio-medial junction. The level of myosin light chain phosphorylation during perfusion with 20 mM K⁺ was 15% (Fig. 4).

After either type of response to 20 mM K⁺, a change in perfusion to normal Krebs solution caused a decline in both force and [Ca²⁺], which brought them towards their original resting values. In none of the experiments did we observe a dramatic drop in the light level, relative to force, upon changing from 109 to 20 mM K⁺, as had been observed when aequorin was used as a Ca²⁺-indicator (Rembold and Murphy, 1986). Essentially the same results were obtained (n = 4) in

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**Figure 4.** (A) [Ca²⁺] and peak force, and (B) time course of myosin light chain phosphorylation in the pulmonary artery (diamonds) and the ileum (circles) during superfusion with 109 mM [K⁺]o measured in two different sets of experiments. The two data sets on the right side in B show the phosphorylation level after 15 min of superfusion with 20 mM [K⁺]o preceded by 15 min with 109 mM [K⁺]o.
experiments conducted at 37°C (data not shown). However, we performed most of the experiments at room temperature to reduce the leakage of fura-2 from the cells. Reperfusion with 109 mM K⁺, after 30 min in 20 mM K⁺, caused the fluorescence to rise to 90.5 ± 6%, and the tension to rise to 112 ± 5% (n = 6), after which both signals declined to, respectively, 75 ± 5% and 101 ± 9%.

The contractions described above were activated primarily by Ca²⁺, and the maintained Ca²⁺ signals were not due to a fluorescence artifact, as shown by the effect of Ca²⁺ entry blockers; when verapamil (10⁻⁵ M) (Fig. 6) was added while the force was maintained in the 20-mM K⁺ solution, the fluorescence ratio and the force declined towards resting levels (n = 5).

We also wished to determine whether quick releases were associated with detectable changes of the [Ca²⁺]. When the quick release was applied either during force development or after 30 min of incubation in high K⁺ no changes in the [Ca²⁺].
could be observed, despite the decrease and subsequent redevelopment of force (see Fig. 8). Restretching the strips to the “prerelease” levels restored the original level of force with, again, no detectable effect on [Ca²⁺].

Phasic Smooth Muscle: Ileum

Resting [Ca²⁺], in ileal smooth muscle was 79 ± 6 nM (24 measurements from 18 muscle strips). During superfusion with high K⁺ (109 mM), fluorescence and tension reached their respective maxima within 1 min. The initial Ca²⁺ spike (374 ± 46 nM) was accompanied by an early force transient, which has been described by Himpens and Somlyo (1988) (Fig. 9). Thereafter, the [Ca²⁺], declined to obtain a value of 229 ± 16 nM after 30 min. The tension frequently underwent a second increase (see After-contraction, Himpens and Somlyo, 1988) after its initial peak. After 30 min of stimulation with 109 mM [K⁺]₀, force declined to 57 ± 7% (n = 6) of its initial peak value.

Phosphorylation at rest was 7% (Fig. 4), and was between 7 and 10% when measured between 30 s and 30 min from the onset of superfusion with 109 mM [K⁺]₀; no significant change could be detected at any time during this interval. The absence of a significant increase in phosphorylation was not due to any functional impairment, as indicated by the normal peak force (2.6 x 10⁵ ± 0.7 N/m²) reached.

Figure 7. The two types of peak fluorescence response and contraction elicited in strips of pulmonary artery by changing perfusion from 109 to 20 mM K⁺. The bars show the magnitude of the fura-2 fluorescence (crosshatch) and tension (diagonal) responses, normalized to the values obtained during the maintained exposure to the 109 mM [K⁺]₀ solution, for the initial response to the 109 mM [K⁺]₀ superfusion.

Figure 8. The effect of a quick release during force development and during the maintained force induced by high K⁺ in the pulmonary artery. Neither the release (triangle) nor the restretch (asterisk) had an effect on [Ca²⁺]. Both tension and [Ca²⁺] fell when [K⁺]₀ was lowered at the end of the experiment.
The failure to detect significant phosphorylation with 109 mM [K+]o at or after 30 s of depolarization did not eliminate the possibility of an undetected early and very rapid phosphorylation transient. Therefore, we measured phosphorylation in four additional strips between 2–3 s after depolarization with 109 mM [K+]o. In two, there was a significant increase to 30%. We also considered the possibility that the net phosphorylation measured during a rapid phosphorylation/dephosphorylation transient could be reduced by asynchronous activation due to diffusion of [K+]o. To minimize as much as is possible such a diffusional delay, muscles were depolarized by a solution containing 140 mM K⁺. This higher concentration of the depolarizing solution is expected to provide a more synchronous activation. These experiments (Fig. 10) revealed significant light chain phosphorylation up to a maximum of 50% within 2–4 s after exposure to 140 mM [K+]o. The maximum force was reached in 8–12 s, while the phosphorylation was already declining. By 30 s, the phosphorylation (12%) was close to basal values, as had been the case during depolarization with 109 mM K⁺.

In another series of experiments (n = 20), the effects of reducing the external [K⁺] from 109 to 20 mM were determined. The fluorescence (Fig. 11) declined after 15 min of superfusion with high K⁺ to 56 ± 3% of its initial peak value. After adding 20 mM K⁺, there was a small transient fall to 49 ± 2% of the peak fluorescence value. The fluorescence then rose again to reach a level that, after 20 min of incubation, was 86 ± 6% of the plateau value at 109 mM K⁺. The phosphorylation values were not significantly different from resting levels (7.5%). Tension declined after 15 min in 109 mM K⁺ to 51 ± 4% of the peak value. Upon adding 20 mM K⁺, tension dropped to 30 ± 4% of its reference value (n = 20).

When the superfusion was changed again from 20 to 109 mM [K+]o (n = 8) (data not shown), fluorescence and force initially rose to 81.5 ± 4.5% and 80 ± 7.5%, respectively, of their initial peak values, and then declined to 52 ± 6% and 40 ± 6% during maintained incubation.
When, instead of 109 mM K\(^+\), 10\(^{-5}\) M verapamil was added to the 20-mM K\(^+\) solution for 15 min, [Ca\(^{2+}\)], and force fell below their respective resting levels (Himpens and Somlyo, 1988) (Fig. 11).

Finally, when a quick release was applied during either the early or the latter phase of the contraction, no changes in [Ca\(^{2+}\)], were observed (Fig. 12). There was
no force redevelopment after the quick release at 15 min. As in the case of the pulmonary artery, the restretching had no effect on [Ca$^{2+}$].

**DISCUSSION**

The most general results of this study describe the relationship between [Ca$^{2+}$], myosin light chain phosphorylation, and tension during prolonged depolarization with high K$^+$, and the parallel effects of changes in extracellular K$^+$ on these parameters. The most interesting result is the very rapid myosin light chain phosphorylation transient found in contracting ileum.

Reduction of [K$^+$]$\text{a}$ from 109 to 20 mM caused either a parallel decline of both [Ca$^{2+}$], and tension or no change in either of these parameters. These results differ from those of Rembold and Murphy (1986), who, using aequorin, found that reduction of [K$^+$]$\text{a}$ from 109 to 20 mM resulted in a disproportionately greater reduction in [Ca$^{2+}$], than tension in swine carotid arterial smooth muscle. The fall in tension caused by the reduction of [K$^+$]$\text{a}$ was disproportionately greater than the fall in [Ca$^{2+}$], in the ileum (Fig. 11), but not in the pulmonary artery; this could reflect a steeper pCa-tension curve of the ileum. The higher levels of [Ca$^{2+}$], and tension reached when perfusion with 20 mM [K$^+$]$\text{a}$ was preceded by 109 mM, compared with 5.9 mM [K$^+$]$\text{a}$ solution, may reflect incomplete repolarization of muscles, but this question was not explored any further.

*Comparison of Contractile Responses of Tonic (Pulmonary Artery) and Phasic (Ileum) Smooth Muscle*

The major differences in the contractile responses of these two smooth muscles to high K$^+$ were that in the ileum the initial phasic force transient was followed by a decline in tension, while in the pulmonary artery smooth muscle there was a continuous increase in tonic tension during prolonged depolarization (cf. Figs. 3 and 9), while [Ca$^{2+}$], declined. (For a discussion of the “after contraction” effects in ileal
smooth muscle see Himpens and Somlyo, 1988.) The absence of an initial "force spike" in pulmonary artery was clearly not due to diffusional effects, because this would have similarly affected the [Ca\(^{2+}\)] transient evoked by depolarization with high K\(^+\). The new information, revealed by the time course of the Ca\(^{2+}\)-sensitive fura-2 fluorescence ratio, is that the above differences in the force response of the two types of smooth muscle were not primarily due to different time courses in [Ca\(^{2+}\)]. The time courses of the latter were similar in both smooth muscles, consisting of an initial peak followed by decline to an elevated value that remained stable during the last 10 min of superfusion with 109 mM K\(^+\). The steady state value of [Ca\(^{2+}\)], was slightly, but significantly (P < 0.05), higher in the pulmonary artery (289 nM) than in the ileum (229 nM) and may have contributed to the longer, albeit slight, persistence of active force recovery in the pulmonary artery. The possibility that a time-dependent inactivation of Ca\(^{2+}\) permeability is less pronounced in tonic than in phasic smooth muscle has been previously considered (Somlyo and Somlyo, 1969).

**Myosin Light Chain Phosphorylation in Pulmonary Artery and Its Unexpectedly Low Level during Force Maintenance in the Ileum**

Myosin phosphorylation and tension had to be measured in a separate set of smooth muscles from those used for [Ca\(^{2+}\)], and tension measurements, because it was not technically feasible to rapidly freeze smooth muscles mounted in the apparatus designed for detecting fura-2 fluorescence. The rates of solution change were faster in the experiments designed to measure myosin light chain phosphorylation. Therefore, during the initial few seconds of activation the time course of [Ca\(^{2+}\)], and phosphorylation cannot be correlated.

After depolarization of pulmonary artery smooth muscle, myosin light chain phosphorylation rose from a resting value of <5%, to a peak value of 50% at 90 s, and declined to a steady state value of 23% by 30 min; this time course is qualitatively similar to that seen in a variety of smooth muscles in numerous laboratories (e.g., swine carotid: Dillon et al., 1981; trachealis: Kamm and Stull, 1985). Minor differences in the absolute values of resting, peak, or maintained levels could reflect small differences in experimental conditions, techniques of measuring myosin light chain phosphorylation, or true tissue-specific variations.

The very transient, rapid time course of the myosin light phosphorylation during stimulation of ileal smooth muscle strips was unexpected. The rapid phosphorylation transient nearly escaped detection since the myosin light chain phosphorylation levels were close to basal values as early as 30 s after activation with 109 mM [K\(^+\)]. To our knowledge, this is the first example in intact smooth muscle of an extremely rapid phosphorylation/dephosphorylation transient, followed by the maintenance of force at near-basal levels of phosphorylation.

The phosphorylation measurements were made on tissues that were immersed in depolarizing solution, rather than on those perfused with high K\(^+\) as was the case in the [Ca\(^{2+}\)], measurements. The faster solution exchange in the immersion would be expected to result in faster activation. Therefore, in an attempt to activate the tissue more rapidly with perfusion during [Ca\(^{2+}\)], measurements, we performed additional experiments in which perfusion was conducted at the maximum pump rate attaina-
ble and with volumes of solutions whose cost was prohibitive to be used routinely. In four such experiments, during rapid perfusion with 140 mM K+, the time to peak for the rise in [Ca²⁺], was 7 s, while force rose to a peak at 8 s. Although these results still represent a slower activation than that attained during the measurements of light chain phosphorylation, they suggest that the time courses of the rise in [Ca²⁺], and myosin light chain phosphorylation are similar.

We cannot exclude the possibility that the maintained force was accompanied by small increases of the myosin light chain phosphorylation, which, with current methods, cannot be distinguished from basal levels. However, in view of the observed steep relationship between phosphorylation and tension in steady state conditions (Ratz and Murphy, 1987), it is possible that small increases in phosphorylation that went undetected by these methods, in conjunction with cooperativity (see below), can contribute to force maintenance. In intact smooth muscle, very low (basal) levels of phosphorylation during force maintenance have been observed (Silver and Stull, 1984) only during prolonged (120 min) muscarinic stimulation. In skinned smooth muscle, force in the absence of measurable myosin light chain phosphorylation has also been observed (Cassidy et al., 1979; Wagner and Ruegg, 1986). However, the very transient nature of the phosphorylation in intact ileum (present study) raises the question whether rapid and transient increases of phosphorylation also preceded force development in skinned smooth muscles, but were undetected due to lack of early sampling. It should also be noted that not all phasic smooth muscles show the basal level of myosin phosphorylation, as does the ileum, during force maintenance. For example, in the *Taenia coli*, increased myosin light chain phosphorylation (30%) could be observed during force maintenance (Siegman et al., 1984).

The majority of studies suggests that contraction of smooth muscle is initiated by the myosin light chain phosphorylation that subsequently declines from its initial peak to lower levels. The latter may rapidly approach basal values, as we have shown here in the case of ileal smooth muscle. The possibility of high rates of myosin light chain dephosphorylation during the cross-bridge cycles has been addressed by others (Driska, 1986; Hai and Murphy, 1988). The unusually low level of light chain phosphorylation found within 30 s after the initiation of contraction in ileal smooth muscle, despite the presence of maintained elevated levels of [Ca²⁺], (present study), suggests that in ileum the ratio of the myosin light chain phosphatase/light chain kinase activity could be increased within seconds after peak force development. These results also demonstrate that the relationship between [Ca²⁺], and phosphorylation is not unique, but may vary from smooth muscle type to smooth muscle type, depending on their respective enzymatic activities.

Cooperative interactions between phosphorylated and nonphosphorylated cross-bridges could be an alternative to (or complement) Murphy’s “latch” model (Murphy et al., 1983; Hai and Murphy, 1988) for the purpose of explaining the high force maintenance during declining but still elevated [Ca²⁺], and low myosin phosphorylation. In such a model, the presence of only a few phosphorylated cross-bridges attached to the thin filament might permit attachment and force development by nonphosphorylated crossbridges. Cooperativity among crossbridges in smooth muscle is indicated by the fact that rigor bridges can facilitate the attach-
ment of and force development by nonphosphorylated crossbridges in the absence of Ca\(^{2+}\) (Somlyo et al., 1988). Such cooperativity could also account for the slow continued rise in force in the pulmonary artery during declining [Ca\(^{2+}\)], and myosin light chain phosphorylation, although we cannot exclude other possibilities like realignment of cells or "minisarcomeres" (small groups of myosin and actin filaments associated with a pair of dense bodies; Ashton et al., 1975) as seen in skeletal muscle (Edman et al., 1978). The lack of sustained maintenance of force in the ileum during depolarization with high K\(^{+}\) might result from a low degree of cooperative force development by unphosphorylated crossbridges, which would be due to the small number of phosphorylated crossbridges. A definitive resolution of some of these questions will require more sensitive methods for measuring very small changes in myosin light chain phosphorylation and, if our hypothesis concerning cooperativity is correct, an estimate of the distribution and minimum number of attached (phosphorylated or rigor) crossbridges required for cooperativity.

In ileal smooth muscle, the complete failure of force redevelopment after a quick release may reflect the low (basal) level of myosin light chain phosphorylation. On the other hand, the maintained elevation of myosin light chain phosphorylation in pulmonary artery may account for the partial active redevelopment of force after the quick release shown in Fig. 8. In any case, the quantitative relationship between myosin light chain phosphorylation and responses to mechanical perturbations remains to be determined in these muscles. The lack of effect of a change in length on [Ca\(^{2+}\)], contrasts with the change in cytoplasmic Ca\(^{2+}\) observed during tetanus in barnacle (Gordon and Ridgway, 1987), cardiac muscle (Allen and Kurihara, 1982), and in frog striated muscles (Checchi et al., 1984) that are regulated by Ca\(^{2+}\) binding to troponin (Weber and Murray, 1973).

B. Himpens is a recipient of Fogarty International Fellowship 1 F05 TW04004. This work has been supported by grant HL15835 to the Pennsylvania Muscle Institute from the National Heart, Lung and Blood Institute. B. Himpens and G. Matthijs are research assistants of the National Fonds voor Wetenschappelijk Onderzoek (Belgium).

We thank Dr. E. L. Buhle, Jr. for his help with the computer programs and Ms. C. Hirschfeld for help with the experiments. The outstanding technical contributions of Mrs. M. Tokito and Mrs. L. Plasterer are also gratefully acknowledged.

Original version received 21 December 1987 and accepted version received 14 July 1988.

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