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Mechanical and biochemical characterization of the contraction elicited by a calcium-independent myosin light chain kinase in chemically skinned smooth muscle

by U. Mrwa, K. Güth, J.C. Rüegg, R.J. Paul, S. Boström, R. Barsotti and D. Hartshorne

II. Physiologisches Institut, Universität Heidelberg, Heidelberg (FRG), Department of Physiology, University of Cincinnati, Cincinnati (Ohio 45267-0576, USA), and III Dept. of Nutrition and Food Science, University of Arizona, Tucson (Arizona 85721, USA)

Summary. The contraction induced by a Ca^{2+}-independent myosin light chain kinase (MLCK-) was characterized in terms of isometric force (F_o), immediate elastic recoil (SE), unloaded shortening velocity (V_{us}), shortening under a constant load and ATPase activity of chemically skinned smooth muscle preparations. These parameters were compared to those measured in a Ca^{2+}-induced contraction to assess the nature of cross bridge interaction in the MLCK-induced contraction. F_o developed in chicken gizzard fibers as well as SE were similar in contractions elicited by either agent. V_{us} in the contraction induced by MLCK-(0.36 mg/ml) was similar though averaged 39.3 ± 8.9% less than V_{us} induced by Ca^{2+} (1.6×10^{-6} M) in the control fibers. Addition of Ca^{2+} (1.6×10^{-6} M) to a contraction induced by MLCK- resulted in small increases in both F_o and V_{us}. Shortening under a constant load was similar for both types of contractions. The contraction induced by MLCK- was accompanied by an increased rate of ATP hydrolysis. The MLCK-induced contraction is thus kinetically similar though not identical to a contraction induced by Ca^{2+}. We conclude that with respect to actin-myosin interaction, MLCK- and Ca^{2+}-induced contractions are similar.

Key words. Smooth muscle; calcium; myosin light chain kinase; regulation of contraction; ATPase; mechanics.

Introduction

Despite intensive studies on the Ca^{2+}-dependent regulation of the smooth muscle contractile apparatus, there is still controversy about the underlying control mechanism(s)[5,19]. One widely held theory is that phosphorylation of the 20,000-dalton light chains of myosin is required for activation of the contractile system[14]. Phosphorylation is catalyzed by a myosin light chain kinase (MLCK) and the Ca^{2+}-dependence of the system is attributable to calmodulin which is required for kinase activity. An alternative theory is that regulation is achieved via a phosphorylation-independent mechanism termed leiotonin[9]. The Ca^{2+}-target in this system is not calmodulin but a protein located on the thin filaments termed leiotonin C. A more frequently considered possibility is that alternative mechanisms may coexist and may function independently or cooperatively[7,8,19]. Usually myosin phosphorylation is considered as one of these mechanisms. In view of the possible involvements of multiple mechanism of Ca^{2+} regulation our initial question is whether or not the phosphorylation of myosin is a component of the regulatory mechanism in smooth muscle and secondly whether one or more mechanisms can be identified?

One way to assess the role of myosin phosphorylation in smooth muscle is to use a Ca^{2+}-independent MLCK (MLCK-) which can be produced by limited proteolysis of the native enzyme[4,18]. Since light chain phosphorylation can be obtained with this system in the absence of Ca^{2+}, it is possible to study the effects of phosphorylation without interference from other Ca^{2+}-dependent mechanisms. To this end the MLCK- was added to 'chemically skinned' smooth muscle fibers and isometric force measured[17]. From these investigations it was suggested that the dominant event in initiating tension development was myosin phosphorylation. It may be argued, however, that isometric force measurement alone may be insufficient to assess the role of myosin phosphorylation since under non-physiological conditions rigor-like contractions may occur. For this reason we undertook a series of experiments in which the contraction induced by MLCK- was characterized in terms of the mechanical parameters of immediate elastic recoil, unloaded shortening velocity, shortening under constant load and the biochemical parameter of ATPase activity of the fiber. These latter parameters, which are more sensitive to cross bridge cycle rate than measurements of isometric tension, are compared to those in a Ca^{2+}-induced contraction to assess the nature of the MLCK-contraction.

Methods

Skinned smooth muscle fibers from fresh chicken gizzard and from guinea pig taenia coli were prepared as outlined previously[12,16]. Gizzards were incubated at 0–4 °C in the glycerol-containing solution for 24 h, rather than 5 min as was the case for the taenia coli, before being stored in the same solution at −25 °C.

Fibers were bathed in a 'relaxing solution' containing (mM) K^+, 21; Na^+, 36, Mg (total), 10; EGTA, 4; ATP, 7.5; imidazole, 20; Cl^-, 35; azide, 1; and an ATP-regenerating system consisting of 10 mM phosphocreatine and 10 U/ml of creatine phosphokinase; pH of the final solution was 6.7 for taenia coli and 7.0 for gizzard. Fibers were stretched (approximately 5%) to a preload between 0.1 to 0.3 mN which was found to be optimal for isometric force. Contraction was induced by partly replacing EGTA with Ca-EGTA, thereby increasing the free Ca^{2+}. The concentrations of free Ca^{2+} were calculated from the apparent binding constants for EGTA[11].

Unloaded shortening velocity and the series elastic component were determined by the 'slack test' technique[2] as described previously[10]. For the estimation of shortening velocity at constant load the gizzard fibers were mounted horizontally between a force transducer (AME 801, Aksjeselkapet Micro-Electronics) and a servo motor driven lever (Ling), using a cellulose nitrate-based glue. The system can be used in either an isometric or isotonic mode. In the isotonic mode the position of the lever is

electronically controlled by a feedback system. Tension was induced under the appropriate condition, e.g., presence of Ca^{2+} or the MLCK-, and the load then released to 20% F_o. All releases were carried out from the same initial fiber length and at 23 °C. Following the load release the length of the fiber was recorded as a function of time.

The ATPase activity of taenia coli fibers was measured using the linked NADH-coupled optical system as outlined previously[3]. With this system tension, induced by Ca^{2+} or MLCK-, could be correlated to ATP consumption. Taenia coli rather than gizzard fibers were used because of a high 'background' ATPase associated with the latter.

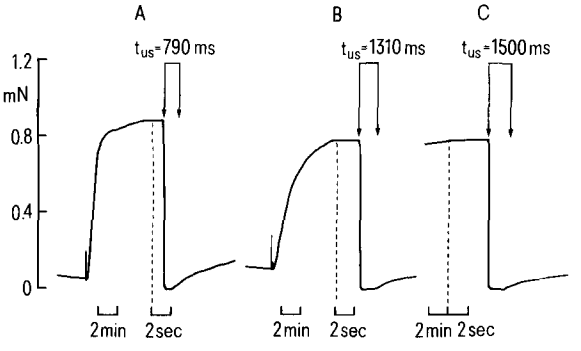

Figure 1. Tracings from chart recorder records showing isometric force vs time for a single chemically skinned chicken gizzard fiber. In panel A contraction was initiated by 1.7×10^{-6} M Ca^{2+} (note surface tension artifact associated with the change of bathing media). After the attainment of maximum force, a rapid decrease in length (20% L_o) was imposed on the muscle (note change in time scale) and the records show the time course of force redevelopment. t_{us}, the duration of time under unloaded conditions, was determined from simultaneous measurements of force on a Nicholet III digital oscilloscope. The fiber was then restretched to l_o and the protocol repeated with a 15% release (not shown). V_{us} and SE were determined from these two determinations of t_{us}. The fiber was then transferred to relaxing solution (4 mM EGTA). After complete relaxation, the fiber length was adjusted to l_o and a contraction elicited by transfer to a bath containing 0.36 mg/ml Ca^{2+}-independent myosin light chain kinase (panel B). The protocol for determination of V_{us} and SE was repeated at the same time following initiation of contraction as in A. Ca^{2+} (1.6×10^{-6} M) was then added to a solution containing MLCK- and measurements for the determination of V_{us} and SE repeated (panel C). All solutions contained 0.5 μM calmodulin, 10 mM phosphocreatine and 10 units/ml creatine phosphokinase. Temperature was 25 °C.

The Ca^{2+}-independent form of the kinase was prepared by the method of Walsh et al.[19] with the following modifications: To a homogeneous preparation of MLCK, purified as described previously[20], a 5-fold excess of calmodulin was added and digestion with α-chymotrypsin (1:50 on a weight basis to the kinase) was carried out for 5 min at 25 °C. Proteolysis was stopped by the addition of lima bean trypsin inhibitor (5-fold excess over the α-chymotrypsin). The digest was applied to a DEAE-Sephacel column equilibrated with 100 mM KCl, 15 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 5% sucrose. The kinase activity was recovered by stepwise elution at 0.26 M KCl. Diisopropyl fluorophosphate was added (0.1 mM final concentration) to each kinase-containing fraction which were pooled and concentrated by Millipore immersible CX-10 ultrafiltration units. The concentrate was dialysed against 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 0.5 mM $MgCl_2$, 5% sucrose, lyophilized and stored at −80 °C. The specific activity of the MLCK- was approximately 0.9 μmol/mg · min^{-1}, and the subunit mol.wt was 85,000–90,000 as determined by SDS electrophoresis. Techniques and procedures associated with the purification and characterization of the kinase were as given previously[18,20]. The concentration of MLCK- used with the skinned fibers was approximately 0.3 mg/ml (~ 3 μM). The ionic content of the lyophilized MLCK was compensated for when added to the fiber solutions.

Results

Unloaded shortening velocity, isometric force and immediate elastic recoil

In order to compare a Ca^{2+}-induced contraction to that induced by the Ca^{2+}-independent MLCK, a protocol was used whereby an initial Ca^{2+}-induced contraction-relaxation cycle was followed by addition of the kinase to the Ca^{2+}-free solution. Reversal of this order of contraction cycles was impractical due to the long time required to wash the MLCK- from the fiber. Because of this fixed temporal order, parallel control experiments in which fibers were repeatedly contracted in Ca^{2+} were performed in order to correct for any changes in F_o and V_{us} ascribable to time or repeated contraction-relaxation cycles. As for all 'chemically skinned' fibers both F_o and V_{us} tend to decrease with time (table). When expressed as a fraction

Comparison of mechanical parameters in contractions induced by Ca^{2+} and MLCK-

Chicken gizzard test	1st contraction 1.7 μM Ca^{2+}			2nd contraction MLCK-			1.7 μM Ca^{2+} + MLCK-		
	F_o* mN	V_{us}* lo/s	SEC* lo	F_o mN	V_{us} lo/s	SEC lo	F_o mN	V_{us} lo/s	SEC lo
$\bar{x}$	1.06	0.171	0.120	0.80	0.085	0.112	0.90	0.097	0.111
SEM	0.14	0.024	0.005	0.10	0.010	0.004	0.14	0.024	0.003
n	5			5			5		

Control	1.7 μM Ca^{2+}			1.7 μM Ca^{2+}		
	F_o	V_{us}	SEC	F_o	V_{us}	SEC
$\bar{x}$	1.19	0.149	0.108	0.92	0.125	0.110
SEM	0.27	0.013	0.004	0.22	0.014	0.002
n	7			7		

*F_o, isometric force; V_{us}, unloaded shortening velocity; SEC, immediate elastic recoil.

of the values in the initial contraction, the values of F_o and V_{us} in the second control contraction, to which comparisons are to be made, averaged (n = 7) 0.75 ± 0.02 and 0.84 ± 0.059, respectively.

As seen in figure 1, the timecourse of the development of force after addition of MLCK- was somewhat slower than that in the presence 1.7 μM Ca^{2+}. However, the timecourse was a function of the concentration of kinase and thus appears to be related to the diffusion of this molecule into the fiber. The steady state force induced by this level of MLCK- (0.3 mg/ml) was equivalent to that induced in the control fiber by 1.7 μM Ca^{2+} (table). Unloaded shortening velocity was measured 6 min after introduction of Ca^{2+} or MLCK- as indicated in figure 1. As can be seen, the redevelopment of force following the rapid length decrease was qualitatively similar for Ca^{2+} and MLCK, although dF/dt was somewhat slower for the latter (as can be judged for example by the total force redeveloped by 8 sec following the release, cf. fig. 1). This is consistent with the data in the table which shows that V_{us} in the contraction induced by MLCK averaged 39.3 ± 8.9% less than V_{us} induced by Ca^{2+} in the control fiber.

It is of interest to note that addition of Ca^{2+} to the contraction induced by MLCK- tended to marginally (~ 10%) increase both F_o and V_{us}. V_{us} under these conditions, however, still remained lower (32%) than that induced by Ca^{2+} in the control fiber. In all cases, differences in the measured values of the series elastic component were small and not statistically significant. Qualitatively similar results were obtained in studies conducted on 3 fibers prepared from taenia coli and in studies on chicken gizzard fibers conducted in the Heidelberg laboratories.

Shortening velocity at constant load

In order to further characterize the contraction induced by MLCK-, shortening under a finite load was studied. Figure 2 shows the time course of the length changes following a release from an isometric contraction to a constant load of 20% F_o. Immediately upon release, there is a rapid shortening corresponding to the series elastic component followed by a much slower component of contractile element shortening. Due to the nonlinear nature of shortening under a load observed for smooth muscle, quantitation of shortening velocities in smooth muscle is somewhat arbitrary[6]. However, as can be seen from Figure 2, constant load shortenings for contractions induced by Ca^{2+}, MLCK-, or MLCK- plus Ca^{2+} are all qualitatively similar.

ATPase activity

A correlation between ATP hydrolysis and tension induced by Ca^{2+} and MLCK- for skinned taenia coli fibers is shown in figure 3. In the control experiments (upper panel) the addition of Ca^{2+} (~ 1.7 μM) elicited a development of tension and this was associated with an increased rate of ATP hydrolysis. The removal of Ca^{2+} induced a relaxation as reflected by a decrease in both tension and ATPase activity. As shown previously[3] maximum ATPase activity preceeds peak tension and also the removal of Ca^{2+} causes a more rapid decrease in ATP hydrolysis than in the decline of tension; i.e., appreciable tension may be recorded at 'background' levels of ATP hydrolysis.

The lower panel of figure 3 shows the correlation of tension and ATP hydrolysis induced by MLCK-. For this

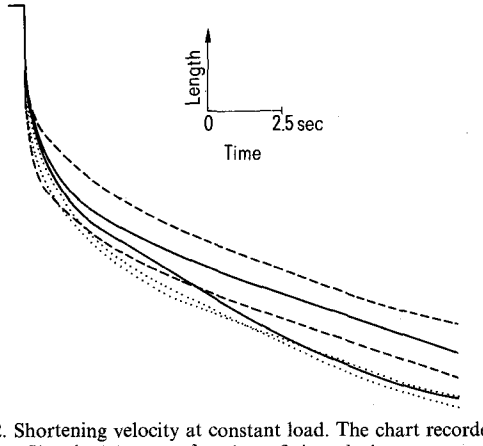

Figure 2. Shortening velocity at constant load. The chart recorder tracings show fiber lengths as a function of time during a constant load shortening (20% F_o) for six fibers. Each line represents the average of three releases performed on one skinned chicken gizzard fiber in the presence of Ca^{2+} (normal contraction solution; dashed line); Ca^{2+}-independent MLCK (0.36 mg/ml) plus 4 mM EGTA (solid line) and Ca^{2+}-independent MLCK (0.3 mg/ml) + Ca^{2+} (dotted line). Each of the fibers had the same initial length and all releases were done from peak isometric force. See 'Methods' for further details.

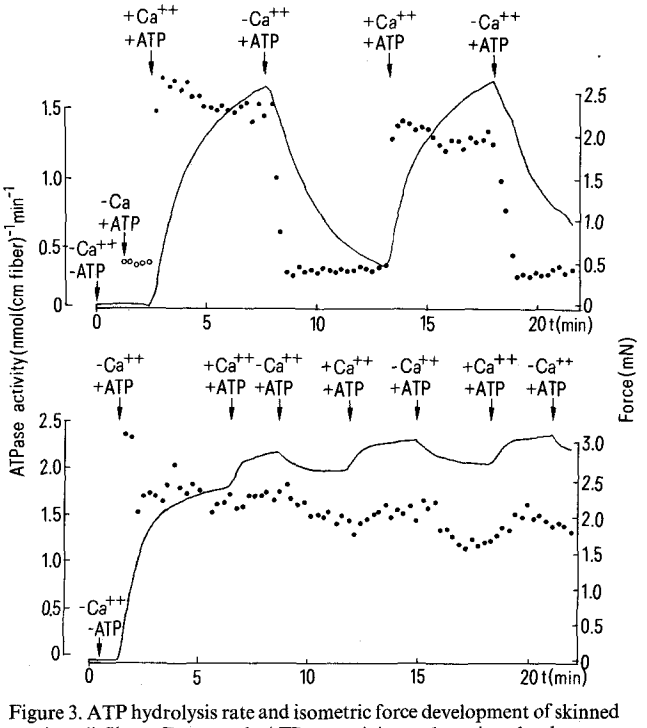

Figure 3. ATP hydrolysis rate and isometric force development of skinned taenia coli fibers. Top panel: ATPase activity and tension development during two (control) cycles of contraction and relaxation. Bottom panel: fiber was incubated in the absence of Ca^{2+} and ATP (relaxing solution minus ATP) in the presence of Ca^{2+}-independent MLCK (0.36 mg/ml) and contraction was initiated by the addition of ATP (7.5 mM). The response of ATPase activity and isometric tension to three sequential cycles of Ca^{2+} addition (contracting solution) and Ca^{2+} removal (relaxing solution) is shown. Two fibers of the same length were used for the two experiments. See 'Methods' for further details.

experiment the taenia coli fiber was preincubated with MLCK- in the absence of ATP, and contraction was initiated by the addition of ATP (in the absence of Ca^{2+}). An increase in both tension and ATP hydrolysis was observed. In this case, however, the increase in the rates of ATP hydrolysis and tension development are not markedly different and whether or not maximum ATPase activity preceeds peak tension cannot be evaluated. Following the initial contraction the fiber was subject to cycles of Ca^{2+} addition and removal. In each case the addition of Ca^{2+} caused a slight increase in both tension and ATP hydrolysis.

Discussion

From the 'slack test' technique one can determine both the unloaded, or maximum shortening velocity and the immediate elastic recoil. The latter is a measure of the series elastic component, part of which has been ascribed to properties within the cross bridge[6]. The SE was found to be the same for all contractions whether induced by Ca^{2+} or MLCK-. Isometric force, in agreement with previous studies[17] was comparable under both conditions. V_{us} and the rate of force development in contractions induced by MLCK- were in a range characteristic of normal cross bridge cycling, but in general were somewhat slower than the cycling rate observed in a 1.7 μM Ca^{2+} contraction. A difference in velocity at the same level of force is not unprecedented in terms of contractions elicited by Ca^{2+}. In a recent report, it was shown that V_{us} could be increased following saturation of the isometric force response by further addition of either Ca^{2+} or calmodulin to skinned smooth muscle[10]. This may underlie the present observations with MLCK. Although the addition of 1.7 μM Ca^{2+} to a contraction induced by MLCK- tended to increase V_{us} and dP/dt, the final values were less (~ 32%) than the control values in the presence of Ca^{2+} alone. Whether this is significant in terms of a slight inhibiting effect of added MLCK- or an independent effect of Ca^{2+} requires further experimentation to resolve.

Theoretically, as unloaded shortening represents an extreme condition (as does isometric force), one might anticipate that differences in mechanical behavior might be exaggerated in contractions at finite loads and velocities. Such constant load experiments in contractions induced by MLCK- were similar to those in contractions induced by Ca^{2+}. Quantitative conclusions were not attempted (due to the nonlinearity in the observed velocity with time) however, no major differences in the time course of muscle length following release can be observed (fig. 2). The pattern to emerge from the measurements of shortening velocity at constant load are in general agreement with the above results. Although of a qualitative nature it appears that the velocities of shortening in either a Ca^{2+} or kinase-induced contraction are similar. In addition, they confirm that the contraction involves actively cycling cross bridges. The latter is also convincingly demonstrated by the correlation of ATPase activity and tension development. In the absence of Ca^{2+}, phosphorylation of myosin causes a marked increase in ATPase activity, which is assumed to correspond to repetitive cross bridge-actin interactions, and this is accompanied by tension development. Clearly Ca^{2+} is not required for this event, although a slight increase in ATPase hydrolysis and tension was observed following the addition of Ca^{2+}. In this work, the earlier studies of Walsh et al.[19] were confirmed in that it has been shown that the level of isometric tension is similar whether induced in the presence or absence of Ca^{2+}. Preliminary experiments on the level of myosin light chain phosphorylation in the presence of MLCK- were undertaken on chicken gizzard fibers. In the EGTA-containing relaxing solution 0.138 ± 0.028 ($n = 5$) mol P_i per mol myosin light chain were found using two-dimensional gel electrophoresis. Under the experimental conditions used in this study, MLCK- induced an increase in phosphorylation to 0.530 ± 0.039 ($n = 5$) which is similar to the value of 0.555 ± 0.015 ($n = 5$) induced by Ca^{2+}. It is, therefore, unlikely that a mechanism other than myosin phosphorylation is involved in the initiation of tension development. In addition, it can be concluded that the contractions induced by MLCK- in the absence of Ca^{2+} involve actively cycling cross bridges and are not dominated by rigor-like bonds. A slight effect of Ca^{2+} was observed in several of the experiments, and although the magnitude of this effect was not sufficient to be conclusive, it does indicate that possibly another mechanism may exist which may modify the cross bridge cycling rate of phosphorylated myosin. This is an interesting possibility that should be explored more fully in the future. It should be pointed out that the experimental protocol chosen for our experiments precludes the collection of data relevant to the potential formation of slowly cycling cross bridges[1, 13, 14]; and any additional regulatory system associated with these would not have been detected[1]. However, in our hands the effect of Ca^{2+} was only slight and the dominant factor determining the cross bridge cycling rate would appear to be myosin phosphorylation.

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Abbreviations

EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Tris, tris (hydroxymethyl) aminomethane; MLCK, myosin light chain kinase; MLCK-, Ca^{2+}-independent form of MLCK; SDS, sodium dodecyl sulfate; V_{us}, velocity unloaded shortening; F_o, isometric force.

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The role of myosin phosphorylation in the contraction-relaxation cycle of smooth muscle[1]

by M. Ikebe and D.J. Hartshorne

Muscle Biology Group, Departments of Biochemistry, Nutrition and Food Science, University of Arizona, Tucson (Arizona 85721, USA)

Summary. Considerable evidence from a variety of experimental procedures indicates that the phosphorylation of myosin is involved in the regulation of contractile activity in smooth muscle. Phosphorylation of the 20,000-dalton myosin light chains is required to initiate crossbridge cycling and this is consistent with the observation that the actin-activated Mg^{2+}-ATPase activity of myosin is phosphorylation-dependent. In the simplest interpretation of this process it may be proposed that phosphorylation acts as an 'on-off' switch. Clearly this cannot explain the observed complexity of smooth muscle contractile behavior and such may imply either that additional mechanisms are involved or that the role of myosin phosphorylation is not fully appreciated. Recently it has been shown that monomeric smooth muscle myosin can exist in a 'folded' and an 'extended' conformation and that each form is characterized by distinct enzymatic properties. Under appropriate solvent conditions phosphorylation of myosin favors the extended conformation. It is tentatively suggest that this, or an analogous, transition might be involved in the regulation of the smooth muscle contractile apparatus, and this possibility is discussed.
Key words. Smooth muscle; regulation; phosphorylation; myosin; conformation; myosin light chain kinase.

The role of myosin phosphorylation in the contraction-relaxation cycle of smooth muscle still is not established. From in-vitro studies, originating with that of Sobieszek[21], it has been shown that phosphorylation of myosin is required for the expression of actin-activated ATPase activity and this has been confirmed in many subsequent reports[2,28]. In addition, numerous investigators have found that tension development in skinned or intact smooth muscle fibers is accompanied by an increase in the level of myosin phosphorylation. Other evidence from a variety of experimental approaches[28] offer further support for the phosphorylation theory. Thus it is reasonable to conclude that phosphorylation of myosin forms at least part of the regulatory mechanism in smooth muscle and the controversial, or unknown, aspects of the regulatory process are concerned with the possible function of alternative or complementary mechanisms.

Evidence cited in favor of an additional process is given by Murphy and co-workers[6] (see article in this volume) who found that the load-bearing capacity of strips of carotid artery was maintained despite a reduction in the level of myosin phosphorylation. From these and other data it was suggested that the phosphorylation of myosin light chains initiates crossbridge cycling (which would be predicted from the phosphorylation-dependent activation of ATPase activity) and that a second mechanism is involved in the maintenance of tension. The latter is