Myosin Phosphorylation and Crossbridge Regulation in Arterial Smooth Muscle

State-of-the-Art Review

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SUMMARY Regulation of vascular resistance is generally explained in terms of neural, hormonal, metabolic, and myogenic factors altering intracellular calcium [Ca++] in vascular smooth muscle. Ca++ acts as a second messenger regulating the number of active crossbridges and force generation by binding to a myofilament regulatory protein. A search for the Ca++-binding regulatory protein in arterial smooth muscle has uncovered what appears to be a new type of regulation. In addition to its interaction with an undefined Ca++-binding site which determines force development, Ca++ stimulates phosphorylation of the crossbridges. Phosphorylated crossbridges cycle more rapidly than dephosphorylated crossbridges in the presence of Ca++. Some known characteristics of the myosin light chain kinase/phosphatase system and the effects of crossbridge phosphorylation on the mechanics of arterial smooth muscle are described. Chronic alterations in this system have potential effects on vascular resistance and merit investigation in studies of arterial smooth muscle from hypertensive animal models. (Hypertension 4 (suppl II): II-3-II-7, 1982)

KEY WORDS • myosin light chain kinase • myosin light chain phosphatase • calmodulin • Ca++-dependent regulatory systems

The calcium [Ca++]-dependent regulatory systems for the contractile proteins in smooth muscle have become the subject of intensive research. This area is also central to an understanding of the role of arterial smooth muscle as the effector regulating vascular resistance. Consequently, some new insights concerning the properties of the contractile system and its molecular control are of interest to students of hypertension or other diseases involving altered vascular smooth muscle function.

This brief commentary has three objectives. The first is to define the subject area by outlining the ways in which vascular resistance is altered by actions of the smooth muscle component of the vessel wall. This will focus on some traditional assumptions about smooth muscle function. The second objective is to describe our recent studies that suggest that myosin phosphorylation is a new type of contractile protein regulation capable of modulating the rates of crossbridge cycling. Current reviews of phosphorylation and regulation in smooth muscle are referenced for those interested in detailed, balanced presentations of the many issues. The third objective is to consider how modulation of crossbridge kinetics may alter smooth muscle function. The focus is on implications for an understanding of the mechanisms involved in the pathogenesis of hypertension and the mechanisms of action for drugs and agonists.

Smooth Muscle and Increased Arterial Resistance

Table 1 schematizes the ways in which arterial smooth muscle may be involved in producing chronic increases in vascular resistance. Hypertension can occur with no alteration in the characteristics of arterial smooth muscle per se. Smooth muscle is the architect of the vessel wall and can be stimulated to induce vessel hypertrophy and structurally produce increases in resistance (table 1, A). Increases in the balance between vasoconstrictor and vasodilator activity resulting from altered neural or hormonal systems will also increase resistance by the normal contractile response of arterial smooth muscle (table 1, B).

While the phenomena listed above are well documented, it has been difficult to unambiguously determine whether arterial smooth muscle is directly involved in the pathology (see Bohr for an extensive literature review and assessment). Changes in mem-
brane properties and the processes involved in excitation-contraction coupling (table I, C) have been documented. Evidence for alterations in the functional capabilities of the contractile apparatus (table I, D) may best be described as equivocal. The final entry in table I (E) is an unexplored possibility arising from studies described below. Assessment of potential changes in vascular smooth muscle cells listed in table I is difficult and must be carried out using isolated tissues, with careful characterization of their mechanical and structural properties. The general approach and basic information about "normal" vascular smooth muscle is covered in several treatises.*

The definition and measurement of contractility and sensitivity are based on three assumptions largely derived from studies of striated muscle. (1) Contractility or maximum force generating capacity is a reflection of the number of active crossbridges generating force additively. (2) Ca\(^{++}\) regulates the number of active crossbridges by acting as a switch: i.e., crossbridges can interact with the thin filament only after Ca\(^{++}\) binds to regulatory sites on the myofibrillar lattice. (3) Variations in the response to Ca\(^{++}\) in terms of the final myoplasmic Ca\(^{++}\) concentration are based on three assumptions largely derived from studies of striated muscle. (1) Light chain phosphorylation is the switch required for crossbridge attachment and cycling; and (2) Ca\(^{++}\) regulates contraction in smooth muscle by combining with calmodulin, followed by formation of the active MLCK-calmodulin-Ca\(^{++}\) complex.*

### Table 1: Postulated Mechanisms to Explain Chronically Increased Vascular Resistance

<table>
<thead>
<tr>
<th>Smooth muscle type</th>
<th>Smooth muscle response</th>
<th>Basis for increased resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>A. Proliferative, synthetic, and secretory response to altered wall stress or unknown factors</td>
<td>Structural changes in wall and reduced lumen</td>
</tr>
<tr>
<td>B. Contractile response to increased neural and/or hormonal system activity</td>
<td>Enhanced tone</td>
<td></td>
</tr>
<tr>
<td>Altered</td>
<td>C. Greater (lesser) response to excitatory (inhibitory) stimuli [increased sensitivity]</td>
<td>More crossbridges activated for given [agonist]</td>
</tr>
<tr>
<td>D. Enhanced force generating capacity [increased contractility]</td>
<td>More or &quot;stronger&quot; crossbridges</td>
<td></td>
</tr>
</tbody>
</table>
| 1. more crossbridges activated for maximum [agonist]  
2. synthesis of more myofibrillar protein  
3. altered myofibrillar proteins | |
| E. Increased crossbridge affinity for actin [decreased shortening velocity] | Lower crossbridge detachment and turnover rates |

**Ca\(^{++}\)-Dependent Regulatory Systems in Smooth Muscle**

Force development by smooth and striated muscle has the same dependence on Ca\(^{++}\) ions.‡ However, the identity of the Ca\(^{++}\)-binding regulatory protein that determines force development by "switching on" the crossbridges remains uncertain. Several myofibrillar candidates have been suggested, including troponin, a new thin filament protein named "leiotonin," and the regulatory light chain of myosin (part of the head of the molecule forming the crossbridge).‡ However, much recent attention has been focused on a novel candidate first discovered in skeletal muscle by Perry and colleagues:§* myosin light chain kinase (MLCK). This enzyme can phosphorylate the regulatory light chains of myosin. Phosphorylation is Ca\(^{++}\)-dependent, as MLCK is only active when associated with the ubiquitous Ca\(^{++}\)-binding regulatory protein, calmodulin. Details of the activation of MLCK and myosin phosphorylation are summarized in several reviews.** Myosin can be dephosphorylated by specific myosin light chain phosphatases (MLCP) present in all types of muscle.**

The crossbridge in skeletal muscle can interact with the thin filament and cycle whether or not it is phosphorylated, and the physiological significance of myosin phosphorylation in skeletal muscle remains controversial.‡ However, the discovery that myosin isolated from smooth muscle only shows significant ATPase activity in the presence of actin when it is phosphorylated led to the hypotheses that: (1) light chain phosphorylation is the switch required for crossbridge attachment and cycling; and (2) Ca\(^{++}\) regulates contraction in smooth muscle by combining with calmodulin, followed by formation of the active MLCK-calmodulin-Ca\(^{++}\) complex.‡* These reg-

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ululatory hypotheses differed from the mechanisms known in skeletal muscle in the sense that Ca++ was postulated to act indirectly to regulate contraction by a covalent modification of myosin. However, the idea was consistent with the well-established switch-like role of other myofibrillar regulatory proteins such as troponin, which determine the number of active cross-bridges. In this scheme, relaxation follows Ca++ sequestration, inactivation of MLCK, and dephosphorylation of myosin by MLCP.

The question we sought to answer was whether the kinase/phosphatase system was the mechanism by which Ca++ regulates contraction in living arterial smooth muscle. The hypothesis was tested on the basis of four predictions. (1) In relaxed living tissue, Ca++ and phosphorylated myosin levels should be very low. (2) A rise in [Ca++] on stimulation should produce an initial increase in myosin phosphorylation prior to force development. (3) The levels of active stress should be proportional to the Ca++ concentration and the level of phosphorylated myosin during maintained contractions. All light chains should be phosphorylated in maximal contractions. (4) The decline of force on relaxation should be preceded by Ca++-sequestration and light chain dephosphorylation. It is technically impossible to measure Ca++ levels in living smooth muscle cells, but the relationships between Ca++ and active stress in these predictions are well established in studies of skinned smooth muscles (and form the foundation of the hypothesis).

Quantitative measurements of the amounts of phosphorylated crossbridges during contraction-relaxation cycles induced by various agonists showed low resting levels of phosphorylation, rapid phosphorylation prior to force development on stimulation, and a fall in phosphorylation to resting levels during relaxation (fig. 1). However, the third prediction was not fulfilled: there was no fixed relationship between the number of crossbridges generating force or active stress and the level of phosphorylation of those crossbridges. We found a precise correlation between crossbridge cycling rates, estimated by measurements of isotonic shortening velocity, and myosin light chain phosphorylation. These studies suggest that two regulatory mechanisms act on the contractile system in smooth muscle. (1) A Ca++-binding regulatory protein that acts as a switch mediating crossbridge interactions must be present. This unidentified protein is demonstrated by the dependence of force on Ca++. However, this regulatory protein is not MLCK, because high levels of force can be maintained or very slowly developed when tissue phosphorylation levels approach the low resting values. Potential candidates for this Ca++-binding protein are leiotonin and the regulatory light chains of myosin itself. (2) The myosin light chain

![Figure 1](https://hyper.ahajournals.org/)

**Figure 1.** Time course (log scale) of isometric stress development, load bearing capacity (LBC), isotonic shortening velocities (at a constant afterload of 0.1 F₀, where F₀ is the maximum force developed at the optimum tissue length, L₀), and myosin light chain (LC) phosphorylation during contraction and relaxation. Data replotted from studies of K⁺-depolarized swine carotid media. Phase I: an unstimulated muscle shows slight tone and low levels of phosphorylation. Phase II: on stimulation, Ca++ influx activates MLCK, and light chain phosphorylation increases in parallel with shortening velocities. Phase III: before active stress or LBC have reached maximum values, phosphorylation (and shortening velocity) begin to decline, indicating a fall in the MLCK/MLCP activity ratio. Phase IV: steady state stress maintenance with significantly reduced crossbridge cycling rates after phosphorylation levels fall to near resting values ("latch"). Phase V: stimulus withdrawal is associated with a rapid decline in residual phosphorylation and ability to shorten. Stress declines very slowly (not too apparent on log scale) as established crossbridges detach or break.
kinase/phosphatase system appears to modulate the actin-myosin interaction. We interpret the dependence of velocity on phosphorylation as the outcome of a population of crossbridges in which phosphorylated crossbridges cycle rapidly, while dephosphorylated crossbridges have a low detachment rate and maintain force with greatly slowed cycling rates (table 2). Somewhat different conclusions have been drawn from experiments in which only isometric force and tissue phosphorylation were measured. The discrepancies appear to be reconcilable when examined in detail. Some biochemical studies of isolated contractile proteins also indicate a dual role for Ca++ in regulation. Our suggestion that the myosin light chain kinase/phosphatase system modulates the kinetics of the crossbridge cycle represents a very different perception of the role of this system than the original regulatory hypothesis. Indeed, the demonstration of a mechanism modulating crossbridge behavior introduces a new element into the basic concepts of regulation of the contractile system in vertebrate muscles. In this instance, studies of living muscles provided critical information not obtainable from biochemical studies of isolated proteins, which cannot discriminate between changes in ATPase activity arising from changes in the numbers of interactions and those due to changes in the rates of interaction.

The dynamic contractile properties of vascular smooth muscle are greatly modified by myosin phosphorylation and the operation of the myosin light chain kinase/phosphatase system, although this is not obvious from measurements of isometric force. The critical question for further work is what determines the level of myosin light chain phosphorylation. This is equivalent to asking what determines the ratio of the activities of MLCK and MLCP. Phosphorylation is undoubtedly triggered on stimulation by the rise in Ca++ and activation of MLCK (fig. 1, Phase II). What, then, is responsible for the fall in phosphorylation and induction of latch (fig. 1, Phase III)? A decline in cellular Ca++ that inactivates the kinase may be the explanation. This hypothesis would require higher cellular Ca++ levels to activate MLCK than needed to permit crossbridge attachment, because force continues to rise and attains a steady value. The observed kinetics may be a function of differences in the rate constants for MLCK and/or MLCP when acting on free or attached crossbridges. Another possibility of physiological interest is that the kinase is inhibited or the phosphatase activated during the course of a tonic contraction. Little is known about factors affecting the activity of the labile phosphatases. However, MLCK can be phosphorylated by protein kinase when stimulated by cyclic AMP. Phosphorylated MLCK has a lower affinity for Ca++-calmodulin. Therefore, increases in cellular cAMP are potentially capable of lowering the activity of MLCK and the level of myosin phosphorylation. Our results show only that increases in cAMP might contribute to the fall in myosin phosphorylation during the course of a sustained contraction. This mechanism could increase the degree of latch but, in our view, cannot be responsible for relaxation, as has been postulated.

Functional Implications of Crossbridge Modulation

It is traditional to consider the output of a muscle in terms of the number of active crossbridges and to quantify this by measurements of isometric force generation, load-bearing capacity, or various estimates of an active state. The evidence for an additional regulatory system that can alter the kinetics of crossbridge cycling has a number of implications for muscle function.

Vascular smooth muscles normally function tonically to resist the load imposed by blood pressure. The latch state allows a great reduction in ATP utilization associated with crossbridge cycling. In fact, the swine carotid smooth muscle is capable of tonically maintaining isometric force with a rate of ATP utilization 300 times lower than that of frog sartorius muscle. This represents a very significant metabolic saving in view of the estimated 2 kg of vascular smooth muscle present in the average man. The energetic advantages of a latch state may be the basis for the characteristics of the MLCK/MLCP system in tonic smooth muscles.

Modulation of crossbridge cycling rates would have one possible functional consequence in a static vascular bed. In the latch state, crossbridges would spend more time in the attached force-generating configuration. This might contribute to the high force-generating capacity of arterial smooth muscle relative to the tissue myosin content. However, resistance of a dynamic system with significant vasomotion might be greatly affected by modulation of crossbridge cycling. The discussion has focused on contraction, but a system that modulates cycling rates, presumably by affecting the rate of crossbridge detachment, would affect relaxation rates. As might be expected, the rate of relaxation of the swine carotid media preparation after agonist withdrawal is much slower when the muscle is in latch (fig. 1) than is the relaxation rate early in a response when the level of phosphorylation is high (W. T. Gerthoffer, unpublished observations). The development of latch might increase average vascular resistance in a dynamic vascular bed.

Many investigators have addressed the difficult question of determining whether the output of the contractile system is altered in vascular smooth muscle from hypertensive animals. These investigations
have assessed output in terms of isometric force development. A question of equal importance, however, is whether the kinetics of crossbridge cycling are pathologically altered with changes in the myosin light chain kinase/phosphatase system. An important first step would be to study the dynamic aspects of contraction in arteriolar smooth muscle preparations from hypertensive animals. Are the magnitude and time course of phosphorylation and isotonic shortening velocity altered during the course of contractions elicited by various agonists? If changes are found, mechanistic hypotheses could be framed and tested.

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