Myosin Light Chain Phosphorylation and Tension Development in Stretch-Activated Arterial Smooth Muscle

Ronald F. Ledvora,1 Michael Bárány,1 and Kate Bárány2, 3

Phosphorylation of the 20 000 Da light chain of myosin in functionally different porcine carotid arteries was determined, with use of two-dimensional gel electrophoresis. Stretching arteries to 1.7 times their resting length resulted in maximal phosphorylation. Intracellular Ca2⁺ was mobilized for stretch-induced light chain phosphorylation. Releasing the stretch from the arteries produced active tension spontaneously, without the participation of any exogenous stimulating agent. Prolonged treatment of arteries with a chelating agent (EGTA) not only abolished stretch-induced phosphorylation, it also prevented the development of active tension when the stretch was released. However, when the EGTA was washed out and the strips were restretched and again released, the stretch-induced phosphorylation and the stretch-release-induced active tension were restored. Evidently, arteries must contain phosphorylated light chain if they are to produce active tension. The myosin light chain became partly dephosphorylated in arteries that developed active tension when the stretch was released, but more than half of the light chains remained phosphorylated. This result suggests that phosphorylation of light chain is involved, not in the generation of active tension, but rather in activation of smooth muscle.

Additional Keyphrases: electrophoresis, two-dimensional · muscle physiology, biochemistry · Ca²⁺, intracellular

The main feature of muscle contraction is the interaction of myosin, actin, and ATP, which causes the myosin-containing thick filaments and the actin-containing thin filaments to slide past each other (1, 2). In vitro, actin activates the ATPase activity of myosin (EC 3.6.1.3); however, in smooth muscle such activation occurs only when myosin is phosphorylated (3–5). In intact smooth muscle, myosin phosphorylation increases during contraction (6–10). Accordingly, myosin phosphorylation has been implicated in the regulation of smooth muscle contraction (for reviews see 11–15).

Stretching arterial smooth muscle induces maximal and reversible phosphorylation of the 20 000 Da light chain of myosin (16, 17). Addition of stimulating agents to the stretched strip neither altered myosin light chain phosphorylation nor elicited contraction (16). Thus, myosin light chain phosphorylation and contraction could be separated. We report here that releasing the stretch produces active tension in arterial smooth muscle spontaneously, without the participation of any exogenous stimulating agent.

Materials and Methods

Helical strips of carotid arteries from freshly slaughtered hogs were prepared and incubated in physiological salt solution containing, per liter, 130 mmol of NaCl, 4.7 mmol of KCl, 1.18 mmol of KH₂PO₄, 1.17 mmol of MgSO₄, 14.9 mmol of NaHCO₃, 1.6 mmol of CaCl₂, 5.5 mmol of glucose, and 0.03 mmol of CaNa₂EDTA, pH 7.4, equilibrated with 95% O₂–5% CO₂, at 37 °C (15). 100 mmol/L K⁺ or 100 μmol/L norepinephrine were utilized as exogenous stimulating agents. Tension was monitored by using a Grass polygraph (Grass Instruments, Quincy, MA 02169) and force transducers.

Figure 1 is a diagram of the muscle incubation chamber. A rest tension equal to about 100 mmHg mean arterial blood pressure was applied to each strip (18), and its length was measured. The strips were then stretched to 1.7 times this "resting length" with a fine-screw manipulator during 20 s. The stretch was immediately partly released for some of the strips, as follows. A strip from which the stretch was to be partly released was attached to the transducer with a double-looped thread. After the strip was stretched, the shorter loop was cut and the strip allowed to shorten. Thus, the extent of the release was determined by the difference between the lengths of the double-loop. In a few experiments, two double-loops were prepared to perform two stretches and two releases with the same strip.

The strips were frozen by immersion in liquid nitrogen, then pulverized and homogenized in a 50 g/L solution of trichloroacetic acid (19). After centrifugation at 40 000 × g, the muscle residue was washed, dissolved, dialyzed, and lyophilized as previously described (20).

![Fig. 1. Muscle incubation chamber](https://example.com/fig1.png)

Two muscles from the same artery were vertically mounted between the stationary hooks and the movable transducers in one water-jacketed chamber. The solution in the chamber was kept at 37 °C and was bubbled with 95% O₂–5% CO₂. The muscle from which the stretch was to be released was attached with a double-looped thread to the transducer. After the strip was stretched, the shorter loop was cut and the strip allowed to shorten. The difference between the lengths of the longer and shorter loops determined the extent of the release.
Two-dimensional gel electrophoresis was performed with 400 and 450 μg of arterial proteins (21), as follows. The freeze-dried samples were dissolved in a solution containing, per liter, 9.5 mol of urea, 20 g of Nonidet-P40 detergent (Bethesda Research Laboratories, Rockville, MD 20850), 0.2 mol of dithiothreitol (DTT), and 20 g of ampholytes, pH 4–6 (Bio-Lyte; Bio-Rad Laboratories, Richmond, CA 94804) and isoelectrofocused in the first dimension on 40 g/L polyacrylamide gels containing, per liter, 9 mol of urea, 20 g of Nonidet-P40, and 20 g of ampholytes, pH 4–6. For electrophoresis in the second dimension we used 150 g/L polyacrylamide gels in 1 g/L SDS and 0.37 mol/L Tris HCl, pH 8.8. The proteins on the gels were stained with Coomassie Blue, then destained, leaving a clear background (21). The stain intensity of the phosphorylated and unphosphorylated forms of the 20,000-Da light chain was measured with a "Quick Scan Jr. TLC Plus" densitometer (Helena Laboratories, Beaumont, TX 77704), which automatically calculated the percentage of total intensity for each spot.

Results

Two-dimensional gel electrophoresis of porcine arterial proteins separated the 20,000-Da light chain from all the other proteins and resolved it into four spots (16). If the strips were 32P-labeled, three of the four spots were radioactive, the least acidic spot being non-radioactive (17). Thus, the first three spots (as one goes from lower to higher pH values) correspond to the phosphorylated forms of the light chain, and the sum of the percentage staining intensities of these three spots gives the percentage phosphorylation of the myosin light chain.

Stretch-Induced Myosin Light Chain Phosphorylation

Figure 2 shows two-dimensional gel electrophoretograms of the proteins of resting and stretched arteries, and the corresponding densitometric scans of the 20,000-Da light chain. The muscle shown on the left in this figure was frozen at resting length (passive force: 25 x g), and the muscle on the right at 1.7 times the resting length (passive force: 200 x g). The percentage stain distribution of the four light chain spots from lower to higher pH values is 5, 12, 25, and 58 in the resting muscle, and 7, 15, 48, and 30 in the stretched muscle. Thus, stretching the muscle increases light chain phosphorylation from 42 to 70%, an increase that is the same as in K+- or norepinephrine-contracted muscles (16). Evidently, stretching per se, even in the absence of stimulating agents, can induce maximal myosin light chain phosphorylation.

Ca2+ is Required for Stretch-Induced Myosin Light Chain Phosphorylation

We have used the strong Ca2+-complexing agent [ethylenebis(oxyethylene)diacetate (EGTA)] to study the role of Ca2+ in myosin light chain phosphorylation of stretched arteries. The gel pictures and densitometric scans of Figure 3 demonstrate the effect of exposure to EGTA. The left frame shows the results for a strip incubated for 4 min with Ca2+-free physiological salt solution containing 1 mmol of EGTA per liter and frozen at resting length; the middle frame shows the results for a strip from the same artery incubated for 4 min with EGTA, then stretched and frozen; and the right frame shows a strip incubated for 20 min with EGTA, then stretched and frozen. The percentage stain distribution of the four light chain spots is 5, 10, 27, and 58 for the resting strip after the 4-min incubation; 7, 13, 45, and 35 for the stretched strip after the 4-min incubation; and 3, 12, 14, and 71 for the stretched strip after the 60-min incubation. The 4-min incubation of the strips with EGTA did not change myosin light chain phosphorylation in either the resting or stretched strips, (cf. the left and middle frames of Figure 3 and the corresponding pictures of Figure 2).

When the 4-min EGTA-treated strip was challenged by K+, neither tension developed nor myosin light chain phosphorylation occurred (the gel-resolved pattern was similar to that for the resting strip shown in the left frame of Figure 3). It is generally accepted that, in K+-contracted muscles, Ca2+ from the extracellular space is utilized, so lack of contraction indicates that the 4-min treatment with EGTA removed the Ca2+ from the extracellular space. When this strip was challenged by norepinephrine, tension developed and myosin light chain phosphorylation increased (the gel-resolved pattern was similar to that for the stretched strip shown in the middle frame of Figure 3). Norepinephrine mobilizes Ca2+ from internal sources, and it elicited myosin light chain phosphorylation and contraction in the 4-min EGTA-treated strip; thus the short exposure to EGTA evidently did not result in the internal Ca2+-stores being lost. Stretching the strip induced myosin light chain phosphorylation in the 4-min EGTA-treated strip to the same extent as did norepinephrine in the unstretched strip; thus we conclude that stretching also mobilizes intracellular Ca2+. In the course of this work we have found that as the exposure to EGTA is prolonged, stretch-induced myosin light chain phosphorylation is decreased. For example, a 60-min exposure to EGTA resulted in drastically decreased phosphorylation, to below the resting level (right frame of Figure 3). It is reasonable to assume that during the 60-min incubation, EGTA removed the Ca2+ from both the extracellular and intracellular spaces of the muscle.

Active Tension Development upon Releasing the Stretch

We have shown previously (16) that stretching arteries to 1.7 times their resting length reversibly prevents development of active tension upon stimulation with K+. This finding implies, in accord with the sliding-filament theory, that there is no overlap between the thick and thin filaments at 1.7 times the resting length. On the other hand, stretching to 1.7 times the resting length induced maximal myosin light chain phosphorylation. If myosin light chain phosphorylation is necessary for activation, and overlap between the thick and thin filaments is necessary for active tension development, it may be expected that releasing the stretch to a length with regions of overlap would elicit spontaneous active tension development. Figure 4 illustrates the spontaneous active tension development when the stretches were released to various extents. To standardize the active tension values, we first contracted the strips by K+-solution at resting length and measured the maximal tension. Subsequently, the K+-solution was washed out by eight rinses with physiological salt solution during 12 min, in the course of which the strips relaxed and myosin light chain phosphorylation returned to the resting value. We then stretched the strips to 1.7 times the resting length and immediately released them to a new length (expressed in percent of resting length on the abscissa of Figure 4). Active tension development instantaneously (expressed in percent of maximal K+-induced tension on the ordinate of Figure 4). Figure 4 shows that the magnitude of active tension development was a function of the release-length. Extrapolation of the length–tension curve indicates that at least 6% of the stretch has to be released to obtain active tension; in other words, the length after the release from 1.7 times the resting length has to be less than 1.66 times the resting length. The data of Figure 4 also show that 100% active tension developed when approximately 30% of the stretch was released.
to a length of 1.48 times the resting length. At greater than 30% releases, spontaneous active tension development was considerably attenuated.

Myosin Light Chain Phosphorylation Is Required for Active Tension Development upon Releasing the Stretch

The active tension development in arteries upon releasing the stretch was markedly influenced by pretreatment with EGTA (Table 1). Strips with two double-loops were incubated in Ca²⁺-free physiological salt solution containing 1 mmol of EGTA per liter for 15, 30, and 60 min, then stretched to 1.7 times the resting length and immediately released to 1.48 times the resting length. The 15-min EGTA-treated strip exhibited spontaneous active tension upon release; the 30- and 60-min EGTA-treated strips did not. Table 1 shows that, concomitant with the loss of active tension development, there is a decrease in the stretch-induced light chain phosphorylation. However, when EGTA was washed out and the strips were restretched and released again, both the stretch-induced phosphorylation and the stretch-release-induced active tension were restored. These experiments suggest that myosin light chain phosphorylation, which is initiated by Ca²⁺, is a prerequisite for smooth muscle contraction.

Reversible Myosin Light Chain Phosphorylation in Stretched and Released Arteries

Figure 5 shows the reversible changes in the level of myosin light chain phosphorylation in functionally different arteries. Myosin light chain is 42% phosphorylated in resting muscles, and it increases to 70% during stretch. The maximal stretch-induced myosin light chain phosphorylation was maintained for as long as 5 s after release of the stretch, independent of the extent of the release or of the magnitude of the spontaneous tension development. Myosin light chain phosphorylation decayed and the dephosphorylation proceeded while spontaneous tension development was
increasing. Myosin light chain phosphorylation decreased to 59% within 30 s after the stretch was released. The rate of dephosphorylation was independent of the extent of the release or of the tension development. Since dephosphorylation and tension development occurred simultaneously, this excludes the possibility that during tension development maximal myosin light chain phosphorylation is required. Figure 5 also shows that a second stretch resulted in the return of myosin light chain phosphorylation to its maximal value, proving reversibility of stretch-induced myosin light chain phosphorylation.

**Discussion**

Bayliss (22) first proposed that constriction of blood vessels may be activated by stretch. Changes in electrical and mechanical activities of vascular smooth muscles during stretch were reported (for a review, see 23). The hysteresis encountered in the length–tension or volume–pressure curves implies an interdependence between the active and passive components of vascular smooth muscles (18). We have shown that stretch alone can induce myosin light chain phosphorylation to the same extent as K⁺- or norepinephrine-induced contraction. Releasing the stretch produced active tension. The extent of active tension depended on the length of the release (Figure 4). Stretch apparently produces an alteration in the structure of muscle, so that the length–active tension curve is shifted to the right, to longer length values. By extrapolating the tension values to zero, we estimated that at least 6% of the stretch has to be released to get active tension. The release of approximately 30% of the stretch resulted in maximal active tension, and larger releases caused attenuated responses.

The length–tension curves for arterial smooth muscle were determined in Murphy's laboratory (24) and imply the sliding-filament theory for smooth muscle. The releases in our experiments provided the necessary overlap between the thick and thin filaments for tension development. However, the overlap between the two sets of filaments is not a sufficient criterion for tension—no active tension was produced in muscles treated with EGTA for 30 min, then stretched and released (Table 1). As shown in Table 1, 30-

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Fig. 3. Two-dimensional electrophoretograms of proteins from arteries at rest or stretched in the presence of 1 mmol of EGTA per liter
Upper panel: gel-staining profiles of the arterial proteins. Lower panel: densitometric scans of the 20 000-Da light chain. Left, strip frozen at resting length after a 4-min exposure to 1 mmol of EGTA per liter of Ca²⁺-free physiological salt solution; middle, strip incubated in EGTA for 4 min, stretched to 1.7 times the resting length and frozen; right, strip incubated in EGTA for 60 min, stretched to 1.7 times the resting length, and frozen. TM, tropomyosin; LC, 20 000-Da light chain

Fig. 4. Spontaneous active tension development as a function of release length in stretch-activated arterial strips
The strips were stretched to 1.7 times their resting length, and were immediately released as described in Materials and Methods. Abscissa: the length after the release, as a percentage of the resting length. Ordinate: spontaneous active tension development expressed as a percentage of the maximal tension of control K⁺-induced contraction. Each point represents a determination with a separate arterial strip. The line drawn through the data from 100% tension to minimal tension was obtained by using a commercial computer program for standard linear-regression analysis.

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Table 1. Relationship between Stretch-Induced Phosphorylation in EGTA-Treated Arteries and Active Tension Development when the Stretch is Released

<table>
<thead>
<tr>
<th>EGTA treatment, min</th>
<th>Stretch-induced phosphorylation</th>
<th>Active tension</th>
<th>After washing out the EGTA*</th>
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<tr>
<td></td>
<td>%</td>
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</tr>
<tr>
<td>60</td>
<td>29</td>
<td>not detectable</td>
<td>68</td>
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*EGTA was washed out with 20 rinses of physiological salt solution during 30 min.

Fig. 5. Reversible myosin light chain phosphorylation in arterial strips at different functional states

Upper panel: demonstrative polygraph tracing of tension for the response of an arterial strip which was stretched to 1.7 times the resting length, released as described in Materials and Methods, and, after spontaneous tension development, restretched. Lower panel: level of myosin light chain phosphorylation at the different states. Each bar represents the mean + SE from 5 to 20 strips.

min treatment with EGTA decreases the extent of light chain phosphorylation, and when EGTA was removed from the muscles by the Ca²⁺-containing physiological salt solution, stretch-induced light chain phosphorylation and stretch-release-induced active tension were restored. Thus, a relationship appears between the phosphorylated state of light chain and the ability of muscle to produce active tension.

It was shown previously that myosin light chain phosphorylation precedes active tension development and decreases while active tension is maintained (17, 25, 26), negating a direct relationship between the magnitude of phosphorylation and tension. The finding that light chain phosphorylation took place in 1.7-times stretched muscles, which were unable to generate tension, clearly separated light-chain phosphorylation from contraction and coupled this phosphorylation with the activation phase of contraction cycle of the arterial smooth muscle (16). The data of the present work revealing light chain dephosphorylation during active tension development (Figure 5) do not support the idea that phosphorylation is involved in the tension-generating proc-

ess. However, the extent of dephosphorylation is relatively small and more than half of the light chains remain phosphorylated during the tension development. This raises the possibility that a "threshold" level of phosphorylation may be needed for the generation of active tension.

The normal function of the cardiovascular system inherently depends on the contractile state of vascular smooth muscle, so it is important to characterize the biochemical mechanisms that are involved in vascular smooth-muscle contraction. A better understanding of the processes during the contraction cycle may provide new avenues for clinical modification of vascular smooth muscle activity in the diseased state.

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