Stretch-induced Phosphorylation of the 20,000-dalton Light Chain of Myosin in Arterial Smooth Muscle*

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Stretching to 1.7 times the resting length of porcine carotid arteries reversibly prevents active tension development by K+ or norepinephrine stimulation. The 20,000-dalton light chain of myosin was maximally phosphorylated in the stretched noncontracting muscles, equal to that in the nonstretched contracting muscles challenged with K+ or norepinephrine. These results show that the contractile event is not a prerequisite for phosphorylation. Furthermore, stretching alone also induced maximal light chain phosphorylation even in the absence of K+ or norepinephrine. The stretch-induced light chain phosphorylation was not affected by exhaustive washing of the muscle with Ca2+-free physiological salt solution, treatment of the muscle with verapamil, or by a short exposure to ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Prolonged EGTA treatment abolished the stretch-induced light chain phosphorylation. All evidence suggests that upon stretch, Ca2+ is released from intracellular sources and this Ca2+ activates the myosin light chain kinase producing phosphorylation of the light chain.

Since the proposal of Bayliss (1) that stretch may activate contraction in arterial blood vessels, changes in the electrical and mechanical activities of vascular smooth muscles which accompany passive changes in muscle length have been measured (2, 3). These studies revealed increased frequency of action potentials and increased contractile activity in response to passive stretch. The biochemical events behind these myogenic reactions remained obscure although it has been tentatively assumed that stretch elicits Ca2+ release (4).

A few years ago, we noticed an increase in the phosphorylation of the 20,000-dalton myosin light chain of porcine carotid arteries stretched to 100 mm Hg mean arterial pressure as compared with that of unstretched arteries (5). Applying recent advances in two-dimensional gel electrophoresis for quantification of light chain phosphorylation in intact muscle (6), we continued our study of the stretch-induced phosphorylation with the aim that such a specific phenomenon may help in a better understanding of the molecular events involved in light chain phosphorylation. To this end, we conducted experiments under conditions in which active tension could not be produced in stretched muscles, so that light chain phosphorylation could be examined independently of contraction.

In this communication, we report maximal light chain phosphorylation in the absence of contraction. The results also show that stretch-induced phosphorylation is initiated by Ca2+ released from intracellular stores.

EXPERIMENTAL PROCEDURES

Porcine carotid arteries were obtained from freshly slaughtered hogs and were transported to the laboratory in ice-chilled physiological salt solution, 130 mM NaCl, 4.7 mM KCl, 1.18 mM CaCl2, 5.5 mM glucose, and 0.03 mM CaNa2 EDTA, pH 7.45 (7). Loosely fat- and adventitia-free carotid strips were prepared from vessel segments of approximately 5 cm in length and 0.4 cm in diameter. Two strips were simultaneously mounted in one incubation chamber and exposed to 60 mM of physiological salt solution equilibrated with a 95% O2, 5% CO2 gas mixture at 37°C. A resting (passive) tension appropriate to each strip's dimensions was applied to simulate 100 mm Hg mean arterial pressure (8). Tension was monitored using a Grass polygraph and force transducers. The strips were incubated at 37°C for at least 20 min prior to use.

Usually, one of the two strips was stretched to 1.7-times the resting length with a fine-screw manipulator over a period of 20 s, while the other strip was kept at the resting length, and both strips were simultaneously frozen by immersion in liquid nitrogen within 30 s. Stretched and unstretched strips were stimulated with K+ challenge solution (100 mM KCl and 35 mM NaCl in the above described physiological salt solution), or NE'-challenge solution (10−4 M NE in the physiological salt solution) and were frozen after 1 and 2 min of stimulation, respectively. No active tension was produced by the stretched strips upon stimulation while the strips with resting tension developed active tension (Table I).

For treatment with Ca2+-free solution, the stretched and unstretched strips were washed 12 times with Ca2+-free physiological salt solution for ½ h. K+ challenge did not result in active tension of the strips; however, after washing with normal physiological salt solution, active tension was developed at rest length in the presence of K+. On the other hand, strips washed with Ca2+-free solution developed tension at resting length upon NE challenge, although no tension could be detected with NE at the stretched length. The strips were treated with 0.2 mM verapamil for 30 min and with 1.0 mM EGTA for 5 or 60 min. Verapamil was dissolved in normal physiological salt solution and EGTA was dissolved in Ca2+-free physiological salt solution.

The frozen muscle strips were pulverized to a powder by percussion using liquid nitrogen chilled mortars and pestles (9) in the cold room of 4°C and the powder was immediately homogenized in ice-jacketed Waring blenders in 5% trichloroacetic acid. After centrifugation at 27,000 x g, the pellets were washed two times with a solution containing 2% trichloroacetic acid and 5 mM KH2PO4, then solubilized with a Brinkman Polytron in 0.25 M Na2HPO4 and 0.45% SDS. The solubilized proteins were dialyzed against 2000 volumes of 0.02% SDS at 25°C overnight. After clarification in the ultracentrifuge, the protein content of the supernatant was determined, and aliquots of the supernatant were freeze dried. Two-dimensional gel electrophoresis was carried out with 400–500 μg of protein as described recently (6). The gels were stained with Coomassie blue and the intensity of staining of the phosphorylated and unphosphorylated P-light chains (10) was measured with a laser scanning densitometer equipped with an automatic integrator (Biomed Instruments).

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RESULTS

We have found that a stretch of 1.70–1.78 times the resting length is required to eliminate the K’-induced tension of porcine carotid arteries. Fig. 1 illustrates such an experiment; an artery under 24 g of resting tension developed 46 g of active tension with the K’ challenge solution. After appropriate washes, the artery was stretched to 1.5 times the resting length exhibiting 80 g passive tension, and it produced 30 g active tension upon K’ challenge. After additional washes, the artery was stretched to 1.7 times the resting length exhibiting 180 g passive tension, and it did not produce active tension upon K’ challenge. However, after further washes and release of stretch, the artery under 24 g of resting tension, produced 24 g active tension upon K’ challenge. Thus, stretching the artery to 1.7 times the resting length reversibly prevented active tension development.

Fig. 2 compares the gel profiles of the P-light chain in arteries at resting length, at 1.7-fold stretched length, and at 1.7-fold stretched length in the presence of K’. Four P-light chain spots are seen in all cases with percentage stain distribution from the acidic to the less acidic side as follows: 4, 9, 14, 50, 31 at stretched length with K’. In 32P-labeled arteries, we have shown that three out of the four spots are phosphorylated and only the least acidic spot is unphosphorylated (11). Furthermore, in highly purified arterial actomyosin we have found the same four P-light chain spots as in the trichloroacetic acid washed arterial residue shown in Fig. 2. This indicates that the four spots are derived from the light chains in the smooth muscle myosin molecule. The extent of phosphorylation of the minor light chain spots is currently investigated in our laboratory. The preliminary data suggest that the light chain is fully phosphorylated in the most acidic spot (peak 1), but only partially phosphorylated in the second acidic spot (peak 2). Accordingly, the extent of P-light chain phosphorylation in this work is expressed as the sum of the percentage stain intensity distribution in peaks 1 and 3 (Fig. 2 and Table I). Thus, in Fig. 2, the phosphorylation increases from 33% at resting length, to 56% at stretched length, and 55% at stretched length with K’ challenge. The data of Fig. 2 demonstrate that full light chain phosphorylation may take place in stretched arterial muscle in normal physiological salt solution or in K’ challenge solution.

Table I shows the effect of stretch on light chain phosphorylation under various conditions. The results have to be compared with those of nonstretched muscles, under about 30 g of resting tension, which exhibit 30% phosphorylated light chain. Upon K’ challenge, such muscles develop 46 g of active tension, while raising the level of phosphorylation to 56%. The passive tension of stretched muscles was 186 g and the light chain phosphorylation was 55%, virtually the same as in nonstretched and K’-challenged muscles. Furthermore, there is no increase in the level of phosphorylation when the stretched muscles are challenged with K’ or NE. The stretch-induced phosphorylation is reversible; upon release of the stretch to a resting tension of 27 g, the phosphorylation decreases to 29%, its original value.

Table I also shows light chain phosphorylation in muscles washed with Ca2+-free physiological salt solution. At resting length, this treatment greatly reduces the phosphorylation with and without K’ challenge, although NE challenge results in a complete phosphorylation. On the other hand, stretched muscles in Ca2+-free physiological salt solution exhibit full light chain phosphorylation even without any challenge, and with K’ or NE challenge. The effect of verapamil, a Ca2+-

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**Fig. 1.** Reversible inhibition of tension development in a stretched porcine carotid artery strip. To an artery 4.5-cm long and 0.5-cm wide, 24 g of resting tension was applied and its length was measured. The strip was contracted with the K’ challenge solution (K’); the ordinate shows the active tension developed. The strip was washed with Ca2+-free physiological salt solution (Wash) until the active tension disappeared, then a final wash of normal Ca2+-containing physiological salt solution was used. The strip was stretched to 1.5 times the resting length exhibiting 80 g of passive tension (1.5 Stretch) and then stimulated with K’. At peak tension development, the washing procedure was repeated. The strip was then stretched to 1.7 times the resting length exhibiting 180 g of passive tension (1.7 Stretch), then stimulated with K’, but active tension was not produced. After 5 min, the washing procedure was repeated and the stretch was released. Subsequently, again 24 g of resting tension was applied to the strip (No Stretch), and contracted with K’. It should be noted that before each K’ addition, the polygraph was readjusted to the same baseline and same sensitivity.

**Fig. 2.** Two-dimensional gel electrophoretic analysis of the distribution of P-light chain in stretched and unstretched porcine carotid arteries. Top shows the gel staining profiles and bottom shows the densitometric tracings of P-light chain. Left, unstretched; center, 1.7 times stretched; right, 1.7 times stretched and K’ challenged. TM, tropomyosin; LC, 20,000-dalton P-light chain.
channel blocker, is also shown in Table I. At resting length, verapamil prevents light chain phosphorylation with or without K+ challenge. However, light chain phosphorylation is manifested in verapamil-treated and stretched arterial strips. Finally, Table I shows that 5-min treatment with EGTA does not affect the stretch-induced phosphorylation, but the 60-min EGTA treatment abolishes it completely.

**DISCUSSION**

The stretch-induced myosin light chain phosphorylation in intact smooth muscle described in the present work adds a new parameter to the conditions of this phosphorylation shown previously under the influence of physiological mediators and pharmacological substances (5, 12-20). It is noteworthy that the same extent of light chain phosphorylation was measured in arteries either stretched to 1.7 times the resting length or stimulated with K+ at the original resting length when the muscle develops active tension. Furthermore, K+ or NE challenge of the stretched muscle does not increase the light chain phosphorylation as compared to that induced by the stretch alone. This indicates that the stretch-induced phosphorylation is maximal.

All evidence suggests that the strength-induced light chain phosphorylation is related to Ca2+ release from an intracellular source. (This Ca2+ activates the myosin light chain kinase and thereby induces the phosphorylation.) Thus, exhaustive washing of the muscle with Ca2+-free physiological salt solution does not eliminate the stretch-induced phosphorylation, and this phosphorylation also withstands verapamil or short EGTA treatment of the muscle (Table I). On the other hand, prolonged EGTA treatment which depletes also the intracellular Ca2+ storage sites, prevents the stretch-induced phosphorylation. As it appears from Table I, stretch-induced phosphorylation parallels that induced by NE in Ca2+-free solution; it is well accepted that NE releases Ca2+ bound inside the vascular smooth muscle (21, 22). Both the sarcoplasmic reticulum and mitochondria were identified as intracellular calcium sites in vascular smooth muscle (23), electron probe analysis may detect whether only one or both of these compartments are involved in the stretch-induced Ca2+ release.

The 1.7-fold stretch of porcine carotid arteries is reversible. Upon releasing the muscle to its original rest length, both the chemical event (phosphorylation) and the mechanical event (loss of active tension upon stimuli) return to their original value (Table I and Fig. 1). Because the stretched muscles did not develop active tension upon stimuli by K+ or NE, it is reasonable to assume that no interaction between actin and myosin filaments took place. (A priori one can not rule out the possibility that the muscle is activated by stretch and it is exerting an active tension, which is not observable because of passive tension is too great to be overcome.) The fact that maximal light chain phosphorylation occurred in the stretched noncontracting muscles indicates that the contractile event of the muscle is not a prerequisite for the phosphorylation of its light chain. It appears that the activation excitation from contraction and differentiating intracellular Ca2+ stores from transmembrane Ca2+ fluxes.

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**REFERENCES**

2. Sparks, H. V. (1964) Circ. Res. 15, Suppl. 1, 254–260

**TABLE I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tension</th>
<th>Distribution of staining intensity in P-light chain</th>
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<tr>
<td></td>
<td>Passive</td>
<td>Active</td>
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<tr>
<td></td>
<td>Peak 1</td>
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<tr>
<td>None</td>
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<td>K+-challenge</td>
<td>30 ± 5</td>
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<td>Stretched, NE challenge</td>
<td>172 ± 16</td>
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