Stretch-induced Myosin Light Chain Phosphorylation and Stretch-release-induced Tension Development in Arterial Smooth Muscle*

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Stretching arteries from resting length to 1.7 times the resting length increased myosin light chain phosphorylation from 40 to 70% in a graded fashion, reaching a plateau at 1.6 times the resting length. When the fully stretched arteries were released, active tension developed without any exogenous stimulating agent. This stretch-release-induced tension approached the same magnitude as that of the control K^+-induced tension. Stretch-induced phosphorylation and the subsequent tension development upon release of stretch were prevented by incubating the arteries in physiological salt solutions containing ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) or chlorpromazine. The inhibition produced by EGTA was reversible. Stretch-induced phosphorylation decreased as a function of time, regardless of whether stretch was maintained, or slackened slowly, or released quickly. While tension developed upon release of stretch, light chain phosphorylation simultaneously decreased. As tension reached and maintained its maximal value, phosphorylation continued to decrease. Thus, light chain phosphorylation is necessary for activation of arterial muscle contraction, but it need not be maintained during tension development or maintenance.

It has been shown repeatedly that phosphorylation of the 20,000-dalton myosin light chain increases during contraction of smooth muscle (1–10). However, the actual role of these changes in light chain phosphorylation during the contraction-relaxation cycle is debated (11–17). Recently, we have shown that stretching arteries to 1.7 times the resting length reversibly prevents active tension development by K^+ stimulation, while phosphorylation reaches the same level in both the stretched noncontracting muscle and the muscle contracting at resting length (18). Furthermore, stretching *per se* elicited maximal light chain phosphorylation even in the absence of K^+ (18). In this paper, we describe that releasing stretched arteries containing phosphorylated light chain leads to active tension development without the addition of an exogenous stimulating agent.

EXPERIMENTAL PROCEDURES

Carotid arteries were obtained from freshly slaughtered hogs. Loose fat and adventitia were cut from the arteries, and helical strips approximately 4.5 cm long and 0.4–0.5 cm wide were prepared. One end of each strip was attached to a stationary hook and the other end to a movable transducer using surgical threads. The tension was monitored on a Grass polygraph. The strips were incubated in 60 ml of physiological salt solution, 130 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 1.6 mM $CaCl_2$, 5.5 mM glucose, and 0.03 mM $CaNa_2EDTA$, pH 7.4, equilibrated with 95% O_2–5% CO_2, at 37 °C (18).

For radioactive experiments, the helical strips were incubated for 1 h in the physiological salt solution containing 2 mCi of carrier-free [^{32}P]orthophosphate. Washing the strips 15 times over a period of 30 min with nonradioactive physiological salt solution was sufficient to remove the ^{32}P from the extracellular space.

An isotonic salt solution containing 100 mM K^+ was used as an exogenous stimulating agent. This produced 50–60 g of active force, corresponding to 0.83–1.0 ×10⁵ N/m^2 of active tension. Some of the strips were incubated for different intervals in physiological salt solution containing 1 mM chlorpromazine or in Ca^{2+}-free physiological salt solution containing 1 mM EGTA.[1]

A resting tension equivalent to 100 mm Hg mean arterial blood pressure was applied to each strip, and the length of the strip at this tension was measured. This length corresponds to the resting length. A fine-screw manipulator was used for stretching the strips (18). The length of the stretched strips was expressed relative to the resting length. The duration of maintained stretch was varied. In some experiments the stretch was released. Quick releases were performed by attaching the strip to the transducer with looped thread. The loop provided an alternate, longer length of thread tied in parallel with the taut, main connection. At a given time after stretching, the taut, main pathway of attachment was severed and the strip shortened. The extent of release was determined by the difference in lengths between the longer and the shorter threads. In a few experiments, two loop arrangements were used in series to perform two stretches and two releases with the same strip.

The muscle strips were frozen during the experiment by immersion in liquid nitrogen, and the strips were pulverized to a powder by percussion using liquid nitrogen-chilled mortars and pestles in the cold room at 4 °C (19). The frozen powder was immediately homogenized in ice-jacketed Waring blenders in 5% trichloroacetic acid. The pellets were washed twice with a solution containing 2% trichloroacetic acid and 5 mM KH_2PO_4, then solubilized with a Brinkman Polytron in 0.25 M Na_2HPO_4 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 then aliquots were lyophilized. Two-dimensional gel electrophoresis (20) was performed with 400 and 450 μg of arterial proteins.

The percentage distribution of the intensity of the stained proteins at the level of the 20,000-dalton light chain was measured with a Helena densitometer equipped with an automatic integrator. In the radioactive experiments, the gels were dried and autoradiographed (21).

RESULTS

The Multiple Forms of the 20,000-Dalton Myosin Light Chain—Four forms of the 20,000-dalton myosin light chain

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate.
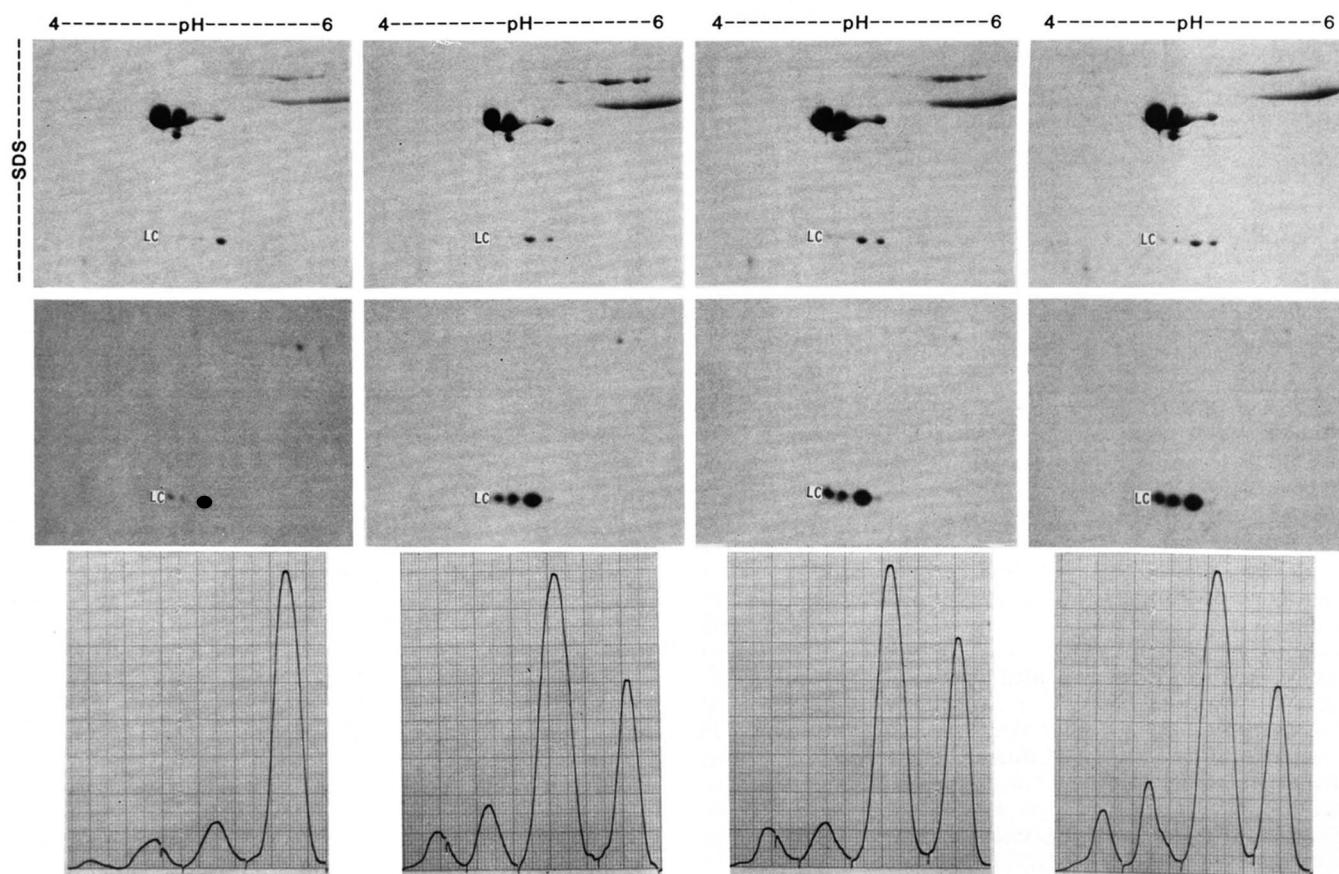
FIG. 1. **Two-dimensional gel electrophoretic analysis of the stimulation of phosphorylation of the 20,000-dalton light chain in porcine carotid arteries.** *Top* shows the gel staining profiles of the arterial proteins, *middle* shows their autoradiograms, and *bottom* shows the densitometric tracings of the 20,000-dalton light chain. Frames refer to arterial strips: *first frame*, at rest; *second frame*, stretched to 1.7 times resting length; *third frame*, K^+-challenged at resting length; *fourth frame*, stretched to 1.7 times resting length and then K^+-challenged. *LC*, 20,000-dalton light chain.

of arterial smooth muscle were revealed by high-resolution two-dimensional gel electrophoresis (18). Changes in the relative distribution of each form were evoked by various stimulations of the intact vascular muscle.

Fig. 1 shows a two-dimensional gel electrophoretic analysis of the muscle proteins from ^{32}P-labeled arterial strips which were resting, stretched, K^+-challenged, or stretched and K^+-challenged. The gel photographs (*top row*) show the four forms of the 20,000-dalton light chain as being clearly separated. The corresponding autoradiograms (*middle row*) of each gel depict the three most acidic forms of the light chain as being phosphorylated. The percentage distribution of the stain in the four forms of the light chain (*bottom row*) is reflected in the four peaks of the scans, referred to as peaks 1, 2, 3, and 4 from the lower to higher pH values. Based on the autoradiograms, light chain phosphorylation is expressed in this paper as the sum of the percentage distribution of the staining intensities in peaks 1, 2, and 3.

The strip in the first frame of Fig. 1 was frozen at rest, 90 min after the resting tension was applied. The percentage distribution of the four forms of the light chain, from lower to higher pH was 3, 10, 16, and 71. Phosphorylation of the light chain was stimulated by stretching a strip to 1.7 times its resting length (second frame of Fig. 1), with a percentage distribution of 5, 13, 56, and 26. Thus, stretching the strip increased the light chain phosphorylation from 29 to 74%. Active contraction of the arterial strip induced by K^+ challenge also increased light chain phosphorylation maximally

(third frame of Fig. 1); the percentage distribution was 7, 11, 54, and 27. Phosphorylation was also maximal in a strip which was stretched to 1.7 times its resting length and then K^+-challenged (fourth frame of Fig. 1); the percentage distribution was 8, 16, 52, and 24.

The autoradiograms show that incorporation of ^{32}P was increased in each of the three spots in the stretched, K^+-challenged, and stretched and K^+-challenged strips, as compared to the resting state. Comparing the effects of stretch to K^+ challenge, stretching specifically increased phosphorylation in the second spot (second and fourth frames in the middle row of Fig. 1), while K^+ challenge specifically increased phosphorylation in the first spot (third and fourth frames). These changes in the intensity of radioactive spots are consistent with the differences in the staining intensities of the light chain spots for each treatment.

Stretch-induced Light Chain Phosphorylation—Fig. 2 illustrates that the stretch-induced light chain phosphorylation is a function of the extent of stretching of the arterial strip. Stretching the strips to different lengths (expressed relative to the resting length) elicited phosphorylation in a graded fashion, reaching a plateau at 1.6 times the resting length. The resting tension (1.0 relative length) equalling 100 mm Hg mean arterial blood pressure was about 20–25 g, and during the continual stretching to 1.7 times the resting length, the applied force gradually increased to a maximum of 250 g. Fig. 2 demonstrates that stretching the arteries is correlated with light chain phosphorylation.
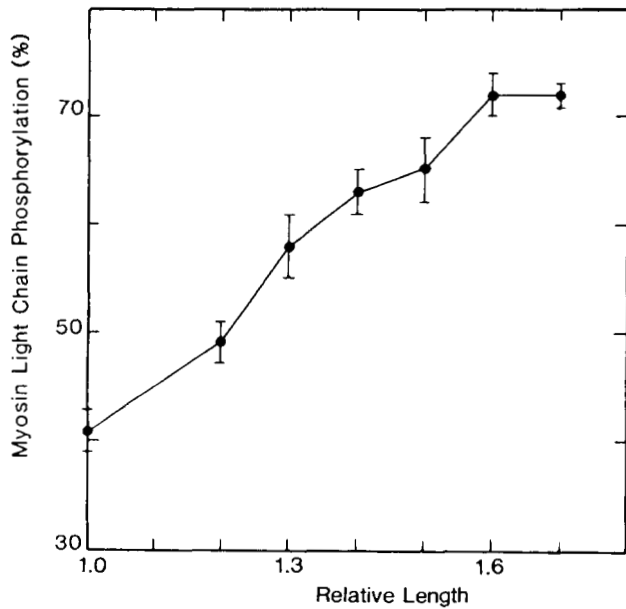
FIG. 2. Myosin light chain phosphorylation as a function of applied stretch in arterial strips. Each arterial strip was stretched and frozen within 3 s after reaching its new length. This new length is expressed relative to its resting length (*abscissa*). Phosphorylation in each strip was determined by two-dimensional gel electrophoresis (*ordinate*). Each point represents the mean ±S.E. from 5 to 10 strips.

Fig. 2 shows an average of 41% light chain phosphorylation at the resting length, a value considerably higher than the 29% shown in frame 1 of Fig. 1 in which the strip has lost most of its initial resting tension during the 90 min incubation/washing period of radioactive experiments. It was previously shown that without resting tension, the phosphorylated light chain content of arterial strips is decreased (1). This finding was confirmed throughout this work: 28–35% phosphorylated light chain was found in strips which were left at slack length. Accordingly, even the limited stretch which is necessary to apply the resting tension induces some light chain phosphorylation.

Stretch-release-induced Tension—We have previously shown that addition of K^+ to arteries stretched to 1.7 times their resting length does not induce active tension (18). These results indicate, according to the sliding filament theory (22, 23), that active tension could not develop due to the absence of an overlap zone between the thick and thin filaments. Therefore, we tested the possibility of tension developing when an overlap zone was provided as a consequence of releasing the stretch.

Fig. 3 shows tracings of representative tension records. As a control, maximal active tension was evoked by K^+ challenge to a strip at resting length. This tension value was taken as 100% (*top* of Fig. 3). The stimulus was removed by washing the strip five times with a physiological salt solution over a 15-min period, during which time the artery relaxed. The strip was then stretched to 1.7 times its resting length and instantly released. Tension development immediately ensued. This stretch-release-induced tension approached the same magnitude as that of the control K^+-induced tension. Generally, 80% of the tension was attained within 1 min, followed by a slow rise or by maintenance of tension for about 30 min. Afterwards, tension gradually declined, reaching the resting value within 1 h, then falling below this value, and approaching zero (not shown in Fig. 3).

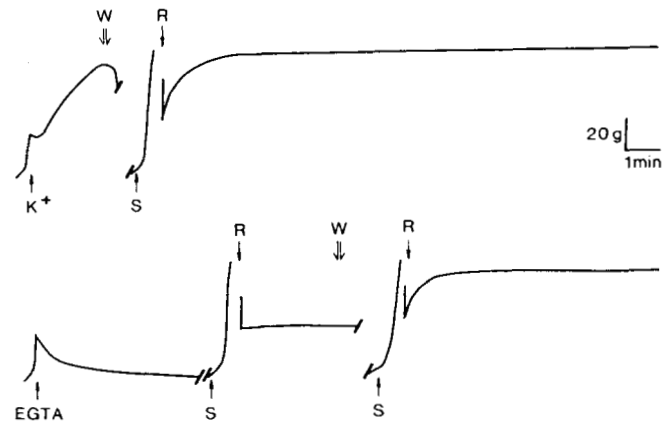The *bottom part* of Fig. 3 illustrates the tension records of

FIG. 3. Stretch-release-induced tension records of arterial strips. *Top*, after adjusting the resting tension of a strip, K^+ challenge solution was added to the bath, and after maximal active tension development the strip was washed (*W*) until tension declined below resting tension. Then the strip was stretched to 1.7 times its resting length over 20 s (*S*); tension during stretching was measured at a 10-fold decreased sensitivity of the polygraph. The stretched strip was released to 1.4 times its resting length (*R*), and the tension was monitored. *Bottom*, after adjusting the resting tension of a strip, 1 mM EGTA in Ca^{2+}-free solution was added to the bath and allowed to react with the strip for 30 min. The strip was then stretched to 1.7 times its resting length (*S*) and was released (*R*). After 2 min, the strip was washed 12 times with physiological salt solution containing Ca^{2+} over 20 min (*W*). The washed strip was stretched a second time (*S*), then released (*R*), and the stretch-release-induced tension was monitored.

a strip which was incubated in a Ca^{2+}-free physiological salt solution containing 1 mM EGTA for 30 min, then stretched to 1.7 times its resting length and instantly released. No stretch-release-induced tension was developed. However, when EGTA was washed out with a physiological salt solution containing Ca^{2+}, and the strip was then restretched and released for the second time, tension development ensued. This experiment indicates that solely providing an overlap zone by releasing the stretch is not sufficient for tension development.

Furthermore, when the strip was incubated for 1 h in a physiological salt solution containing 1 mM chlorpromazine, a calmodulin antagonist (24), then stretched to 1.7 times its resting length and instantly released, little (less than 5 g) or no tension developed. Extensive washing of the strip did not remove the inhibitory action of chlorpromazine, *i.e.* stretching and releasing for a second time did not restore stretch-release-induced tension (not shown).

Decay of Stretch-induced Myosin Light Chain Phosphorylation—Stretch-induced light chain phosphorylation spontaneously decreases over time. Fig. 4 (*top row*) shows two-dimensional electrophoretograms of proteins from arteries stretched to 1.7 times their resting length, then frozen at 30 s (left frame) and 30 min (right frame) after stretching while under passive tension in the stretched state. Below each photograph is the respective scan of the light chain. The percentage distribution of the light chain was 5, 13, 46, and 36 when the stretched artery was frozen after 30 s, and 4, 11, 24, and 61 when the stretched artery was frozen after 30 min. A comparison of these values with those from the second frame of Fig. 1 shows that phosphorylation decreased from 74 to 64% within 30 s and to 39% within 30 min. Fig. 5 shows the decay of phosphorylation under three different sets of experimental conditions. All the strips were stretched to 1.7 times their resting length, resulting in a range of maximal light chain phosphorylation values from 68 to 75%, with the
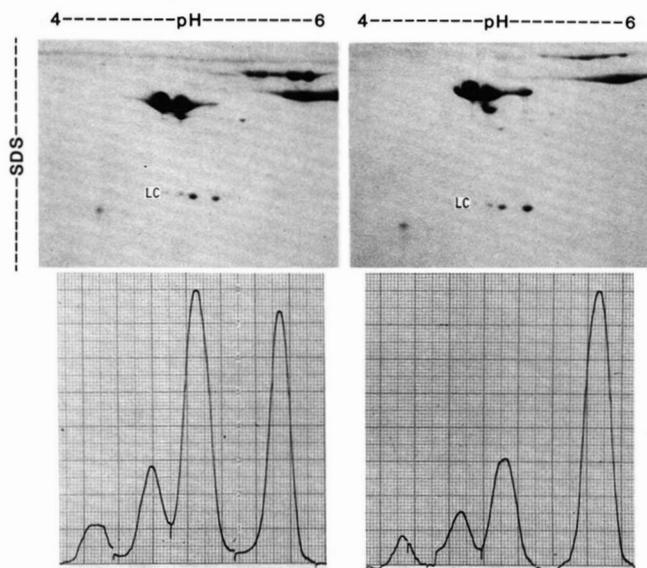
FIG. 4. Two-dimensional gel electrophoretic analysis of myosin light chain phosphorylation in stretched arteries. *Top* shows the gel staining profiles of the proteins in the stretched arteries, and *bottom* shows the densitometric tracings of the 20,000-dalton light chain. *Left frame* refers to an arterial strip frozen 30 s after stretching, and *right frame* represents an arterial strip frozen 30 min after stretching. *LC*, 20,000-dalton light chain.
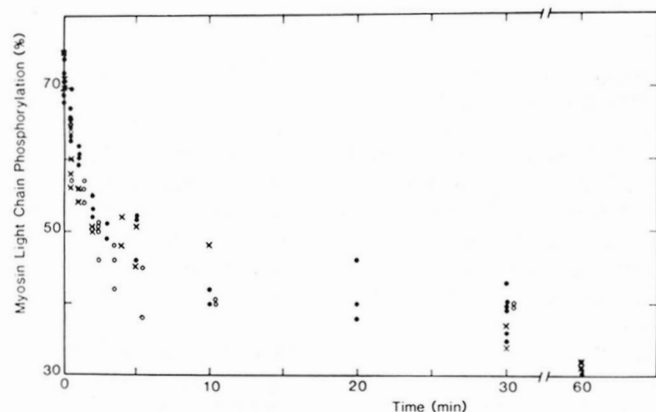

FIG. 5. Decay of stretch-induced myosin light chain phosphorylation in arterial strips. Each strip was stretched to 1.7 times resting length over 20 s, indicated on the *abscissa* as "0" time. Subsequently, each strip was treated in one of the following three ways: 1) kept at the stretch length (●); 2) returned to the resting length over 20 s (○); 3) quick-released to the resting length within 1 s (×). Then, at the time shown on the *abscissa*, each strip was frozen. Each point on the figure corresponds to a separate experiment.

TABLE I

Stretch-release-induced tension and light chain phosphorylation

Strips stretched to 1.7 times their resting length were immediately released to 1.4 times their resting length. The stretch-release-induced tension is related to the 100% value of the K⁺-induced tension.

Time between release of stretch and freezing	Stretch-release-induced tension	Light chain phosphorylation
	%	%
3 s	Development apparent	71 ± 3 ($n = 12$)
30 s	60	59 ± 3 ($n = 20$)
30 min	100	39 ± 3 ($n = 10$)

phosphorylation in these strips was comparable to that of the two previous groups. Accordingly, dephosphorylation of light chain occurred whether or not the stretch on the muscle was maintained, slowly slackened, or quick-released. That is to say, dephosphorylation of light chain proceeded independently of the mechanical state of the muscle.

A comparison of Fig. 5 with Fig. 2 shows that the resting level of light chain phosphorylation, 41% (Fig. 2), is attained in the stretched arteries about 30 min after initiating the stretch (Fig. 5). The level of phosphorylation fell below the resting value after 60 min and further decreased after 90 min (not shown). However, light chain phosphorylation redeveloped when strips were stretched for a second time (not shown), demonstrating the reversibility of the mechanical treatments of the muscle.

Stretch-release-induced Tension and Light Chain Phosphorylation—The extent of tension development following the quick release of fully stretched strips was dependent on the length of the release and the level of light chain phosphorylation. Maximal tension development occurred when the strips were released to lengths between 1.4 and 1.5 times the resting length. When the release was outside this range, tension was attenuated. About 70% light chain phosphorylation was initially required for maximal tension development. When the stretch was released after 60 min, the level of light chain phosphorylation was reduced to approximately 30% (*cf.* Fig. 5), and no significant tension was produced.

Table I shows changes in the phosphate content of light chain during stretch-release-induced tension development. Light chain phosphorylation, on the average, decreased from a maximal value of 71 to 59% during the first 30 s of tension development. Phosphorylation continued to decrease while tension reached and maintained its maximal value. Paradoxically, then, an increase in tension development occurred while the level of light chain phosphorylation decreased. It is of interest to note at this point that Inagaki *et al.* (25) recently reported a correlation between myosin light chain dephosphorylation and enhanced serotonin release from human platelets.

DISCUSSION

Bayliss' (26) proposal that stretch is a stimulus for blood vessel constriction finds its biochemical basis in the results of this work: myosin light chain phosphorylation, a prerequisite for smooth muscle contraction, takes place upon stretching of arterial muscle. This is a novel mechano-chemical coupling; the external work performed on the muscle is transformed into a chemical activation of the contractile protein, myosin.

Stretch-induced myosin light chain phosphorylation has the following characteristics. 1) It is instantaneous. We ob-

average being 71%. One group of strips was frozen at various times after stretching while under passive tension in the stretched state. Light chain phosphorylation decreased to 59–62% within 1 min, to 47–52% after 5 min, then declined at a slower rate to 36–44% after 30 min, and to 31% after 60 min. The second group of stretched strips was returned to resting length with the fine-screw manipulator over a 20-s interval immediately following the stretch. Light chain phosphorylation in these strips decreased as a function of time in a pattern similar to that of the first group of strips, which was frozen in the stretched state. The third group of stretched strips was quick-released, as described under "Experimental Procedures," to resting length within 1 s. The decay of light chain

served this phenomenon at the earliest time of muscle freezing, within a few seconds. 2) It is proportional to the extent of stretch (Fig. 2), as may be expected from a true activator of smooth muscle contraction. At the maximal stretching, 1.6–1.7 times the resting length of porcine carotid arteries, stretching *alone* produces the same maximal (about 70%) light chain phosphorylation as K^+ challenge or stretching and K^+ challenge combined (Fig. 1). 3) It requires Ca^{2+} (Fig. 3 and Ref. 12), suggesting involvement of the myosin light chain kinase in the phosphorylation process. 4) It decays as a function of time (Figs. 4 and 5). 5) It is reversible, *i.e.* light chain phosphorylation, dephosphorylation, and rephosphorylation can be induced by stretch, release, and restretch of arteries.

When released, stretched arteries spontaneously develop tension, apparently imitating the process involved in the regulation of blood pressure. Moreover, the tension produced upon stretch release is of the same magnitude as that of the K^+-induced tension. The stretch-release-induced tension development requires a high level, about 70%, of phosphorylated light chain. When the phosphate content of the light chain is reduced, the stretch-release-induced tension is also diminished. Furthermore, whenever the stretch-induced phosphorylation is inhibited, the stretch-release-induced tension is also repressed; with EGTA the process is reversible (Fig. 3); with chlorpromazine it is irreversible. These data support the notion that the stretch-induced light chain phosphorylation may be significant in contributing to the understanding of the regulation of blood pressure at the molecular level.

Stretch-release-induced tension is accompanied by a partial dephosphorylation of the light chain. In view of the fact that this type of tension resembles that produced by exogenous stimulating agents, the simultaneous increase in tension development and decrease in phosphorylation suggest that light chain phosphorylation cannot be an integral part of the tension development process.

The results presented in this paper indicate that light chain phosphorylation is necessary for the activation of arterial smooth muscle contraction, but it does not have to be maintained during tension development or tension maintenance (6, 8). These observations lead indirectly to the corollary that vascular smooth muscle contraction involves more than one regulatory mechanism (27, 28).

It has been shown recently, *in vitro*, that protein kinase C phosphorylates the 20,000-Da light chain of turkey gizzard smooth muscle heavy meromyosin (29). We are currently investigating the possibility that this enzyme is involved in the stretch-induced phosphorylation of arterial smooth muscle myosin.

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