MYOSIN LIGHT CHAIN PHOSPHORYLATION

IN ARTERIAL SMOOTH MUSCLE

BY

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THESIS

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Vassilis Mougios

ENTITLED MYOSIN LIGHT CHAIN PHOSPHORYLATION IN ARTERIAL SMOOTH MUSCLE

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80173

Τα θεμελια μου στα βουνα

To my roots

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I am grateful to my advisor, Dr. Michael Barany, for teaching me how to do sound and responsible research and for giving me the independence that made working in the laboratory a pleasure. I am equally grateful to Michael Barany for his efforts to have my wife and me accepted into this Department and for his invaluable help during our first year in the United States.

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VM

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LIST OF ABBREVIATIONS

MHC	myosin heavy chain
LC	20-kDa myosin light chain
MLCK	myosin light chain kinase
HMM	heavy meromyosin
Ser-P	phosphoserine
Thr-P	phosphothreonine
pI	isoelectric point
PSS	physiological salt solution
TCA	trichloroacetic acid
SDS	sodium dodecyl sulfate
IEF	isoelectric focusing
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
DTT	dithiothreitol
PMSF	phenylmethylsulfonyl fluoride
Tyr-P	phosphotyrosine

SUMMARY

The 20-kDa myosin light chain (LC) of porcine arterial smooth muscle exhibits multiple isoelectric variants in two-dimensional gels. Upon two-dimensional isoelectric focusing, each variant refocused as a discrete spot and no additional spots were generated, suggesting that the multiple forms are not the result of artifactual charge modification. The same conclusion was reached by pre-electrophoresis of gels in the presence of the reducing agent thioglycolate, which eliminates charge modification caused by oxidation. Thioglycolate failed to eliminate or even change the amount of any LC spot. Completely dephosphorylated LC consists of two forms with isoelectric points of 4.73 and 5.06 and a net charge difference of 2, at a proportion of 15 and 85%, respectively. The two forms were purified from porcine aorta by actomyosin extraction, guanidine.HCl denaturation and ethanol fractionation, preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and preparative isoelectric focusing. Their structures were compared by tryptic peptide mapping and were found to be very similar, but not identical, suggesting that the two polypeptides are isoforms of the LC. The two isoforms had similar phosphorylation patterns in all systems studied. In intact carotid arteries the LC exists mainly in unphosphorylated and monophosphorylated forms, whereas a small

amount (up to 8%) is diphosphorylated. The first phosphorylation site is serine and the second phosphorylation site is threonine. The ratio of phosphoserine to phosphothreonine is approximately 11 in arteries arrested at resting tension or after stimulation with KCl or norepinephrine. The same ratio decreases to approximately 8 upon stretching the arteries to 1.7 times their resting length and is not affected by further stimulation with KCl. The LC pattern of aorta homogenates incubated with ATP is similar to that of stimulated intact arteries. However, the LC pattern of crude aorta actomyosin incubated with ATP shows much higher phosphorylation. Six spots appear in two-dimensional polyacrylamide gels, corresponding to the un-, mono-, di-, and triphosphorylated forms of the two LC isoforms, with partial overlap. These results show that arterial smooth muscle contains protein kinases catalyzing the incorporation of up to 3 mol of phosphate per mol of LC. These kinases not only remain with the crude actomyosin during its preparation, but, by an unknown mechanism, cause much higher phosphorylation than in intact tissue or in tissue homogenate.

I. INTRODUCTION

In the thirty years since the discovery that phoshorylation modifies the activity of glycogen phosphorylase in liver (1) and muscle (2), protein phosphorylation has been recognized as a major regulatory mechanism of biochemical processes at all levels of cellular organization (reviewed in 3 and 4). Muscle has played a central role in this development, because it has been the source not only of the first protein shown to be regulated by phosphorylation, but also of the first well-characterized protein kinase, phosphorylase kinase, and of the reciprocal enzyme, phosphorylase phosphatase. Moreover, muscle was the first source of cyclic AMP-dependent protein kinase (5).

In the 1960's attention was focused on phosphorylation of the soluble muscle enzymes, and it was not until the early 1970's that phosphorylation of myofibrillar proteins was reported. Phosphorylation of myosin light chain was first detected in skeletal muscle (6) and has since been shown in cardiac and smooth muscle as well. Despite many structural similarities between striated and smooth muscle myosins, the effects of myosin phosphorylation are not the same. In striated muscle myosin phosphorylation appears to modulate the interaction of actin and myosin (7), but it is not required for each muscle twitch or heartbeat (8) and has no effect on the actin-activated myosin MgATPase activity (9). In smooth muscle, on

the other hand, phosphorylation and dephosphorylation of myosin parallels the contraction-relaxation cycle, and actin is unable to activate the myosin ATPase unless myosin is phosphorylated (see sections II.B and F). These findings underline the physiological importance of myosin phosphorylation for smooth muscle function and have drawn the attention and efforts of researchers for the past decade.

Muscle contraction can be followed easily and quantitatively. Thus muscle is an ideal model system for studying the relationship between protein phosphorylation and tissue function. Generally, protein phosphorylation can be determined by either of two methods. One is to label the organism or tissue or protein(s) with ^{32}P and measure the incorporation of radioactivity from [γ-^{32}P]ATP into the protein of interest. The second method is to separate the phosphorylated from the unphosphorylated form of the protein, based on their difference in charge, and measure each form. Because the second method does not require the use of radioactivity, it is more convenient for routine work. However, when it was used to study smooth muscle myosin light chain phosphorylation in this and other laboratories, an unusual and, at that time, inexplicable pattern was obtained. My research focused on examining and identifying this pattern. In the course of my work I came upon new and interesting features of the light chain, which provided an answer to the original question, but at the same time opened new areas of investigation. This work is described below.

II. LITERATURE REVIEW

A. Smooth muscle and contraction

Movement is a basic characteristic of the animal kingdom. The movement of limbs or the pumping of the heart powered by striated muscles are the most obvious manifestations of muscle contraction, but there are other muscles which are equally important for survival: these are the smooth muscles.

Smooth muscles surround the hollow cavities of the body: blood vessels, trachea, bladder, uterus, and the various regions of the gut: gizzard, stomach, intestines, and sphincters. Their function is to squeeze the contents; for instance in regulating blood pressure. Smooth muscles are therefore not required to contract quickly, but they must be able to exert tension for prolonged periods. Since smooth muscles are involved in many homeostatic mechanisms, they are mostly under the control of the autonomic nervous system, endocrine secretions, and local hormones.

Smooth muscle cells are generally small and fusiform. They are frequently embedded in large amounts of connective tissue. Neither the tissue nor the individual cells show the characteristic striations of skeletal and cardiac muscle; nevertheless, the contractile apparatus and the mechanism of contraction in smooth muscle is the same as in striated muscle. Contraction is due to the active sliding of thick (myosin) and thin (actin) filaments relative

to each other (10,11). Crossbridges arising from the myosin filaments bind actin and MgATP, the MgATP is hydrolyzed on the enzymic site and the energy released produces a conformational change in the actin-myosin complex resulting in relative movement of the two filaments (12).

The regulation of contraction is a point of clear distinction between striated and smooth muscle. In striated muscle excitation of the plasma membrane results in release of calcium that is stored in an intracellular membrane system, the sarcoplasmic reticulum; the Ca^{2+} released into the cytoplasm allows the interaction of actin and myosin (and therefore contraction) through the regulatory proteins troponin and tropomyosin, both associated with the thin filaments. In smooth muscle, contraction is triggered not only by depolarization of the plasma membrane, but also by neurotransmitters that do not change the membrane potential. Stimulation causes Ca^{2+} flux into the cytoplasm from the sarcoplasmic reticulum or the extracellular space, but just what is the link between Ca^{2+} and contraction has been a matter of controversy and contradiction. Evidence has been presented for myosin filament- and actin filament-linked Ca^{2+} regulation, and supporters of one hypothesis usually preclude the other. Concerning regulation of the thin filaments, two possible mechanisms have been proposed. Ebashi et al. (13) have described a 100-kDa smooth muscle protein, termed leiotonin, which binds and activates a normally inactive actin filament in the presence of Ca^{2+}. Other researchers (14) have

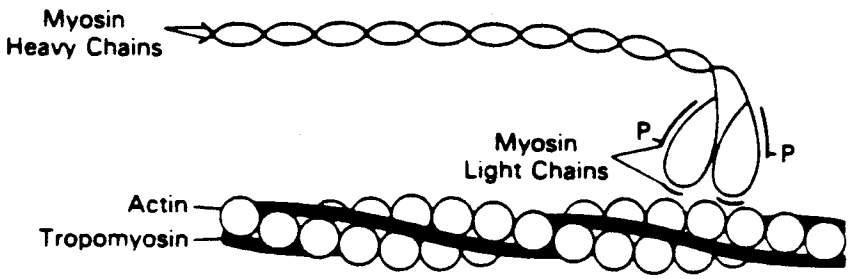
prepared Ca^{2+}-sensitive thin filaments which contain a 120-kDa protein resembling caldesmon, a calmodulin and filamentous actin binding protein of smooth muscle. The model of thick filament-linked regulation, which is the more popular, will be presented in detail in the following sections.

B. Phosphorylation of smooth muscle myosin in vitro

The smooth muscle myosin molecule is morphologically indistinguishable from skeletal or cardiac myosin: it has two globular heads joined by a tail (Figure 1). In the smooth muscle cell the myosin molecules are aggregated into filaments with the globular heads sticking out from the filament surface (11). Myosin is made up from six polypeptide chains: two heavy chains (MHC) of molecular mass 200 kDa and two pairs of light chains of molecular mass 20 kDa and 17 kDa. Each heavy chain constitutes most of one head and half the tail, and one of each light chain type is contained in each head (16).

In 1975 Sobieszek (17) observed a Ca^{2+}-dependent phosphorylation of the 20-kDa myosin light chain (LC) in gizzard actomyosin preparations. The Ca^{2+} dependence of phosphorylation was close to that of the activation of MgATPase activity; phosphorylation preceded activation of the MgATPase and both high MgATPase and phosphorylation persisted after the removal of Ca^{2+}. The observations on gizzard myosin were quickly confirmed (18) and expanded to other smooth muscles such as vas deferens (19), stomach (20), blood vessels (21,22), uterus (23), and placenta (24). The

Figure 1. Diagrammatic representation of the major contractile proteins of smooth muscle. The 20-kDa light chains of myosin can be phosphorylated (P). Reproduced from reference 15.


Myosin
Heavy Chains
Myosin
Light Chains
P
P
Actin
Tropomyosin

complete sequence of the 171 amino acids of gizzard LC has been published (25) and the site of phosphorylation has been located on serine 19 (25,26).

Phosphorylation of the LC has a remarkable effect on the ability of actin to activate the MgATPase activity of smooth muscle myosin. Myosin MgATPase is not affected by the level of light chain phosphorylation in the absence of actin (23,27). The MgATPase activity of unphosphorylated myosin is not activated by actin, but the actin-activated myosin MgATPase increases dramatically upon phosphorylation and becomes maximal when the LC is fully phosphorylated (20-24,27-33). Therefore both myosin phosphorylation and the presence of actin are needed for activation of smooth muscle myosin MgATPase. The increase in the MgATPase activity is reversible, since it decreases upon dephosphorylation of the LC (20,27,32). Also LC phosphorylation is required for superprecipitation of smooth muscle actomyosin (22,23,32), an assay that measures the increase in turbidity of an actomyosin gel when it contracts in the presence of MgATP and Ca^{2+}. These results show that myosin phosphorylation is necessary for the two events that are considered the <u>in vitro</u> equivalent of contraction, namely, ATP hydrolysis and actomyosin superprecipitation. Thus a novel mechanism of regulation of contraction was postulated for smooth muscle. The hypothesis may be stated briefly as follows:

1. The ATPase site of myosin cannot interact with actin unless the LC is phosphorylated.

2. Phosphorylation is catalyzed by a kinase that is activated by Ca^{2+} at concentrations similar to the cytoplasmic Ca^{2+} concentration of stimulated muscle.

3. Dephosphorylation is catalyzed by a phosphatase which is active regardless of Ca^{2+} concentration.

Support to this hypothesis came from the detection of endogenous Ca^{2+}-dependent myosin light chain kinase and Ca^{2+}-independent phosphatase activity in fractionated smooth muscle extracts soon after the discovery of LC phosphorylation (19,20,23,24,28-33). These enzymes have now been purified and characterized:

C. Myosin light chain kinase

Myosin light chain kinase (MLCK) has been purified to homogeneity from gizzards (31,34-36), arteries (37,38), stomach (39), trachea (40), and uterus (41). It consists of two subunits, one of which is the ubiquitous 17-kDa Ca^{2+}-binding protein calmodulin (31,42). The other subunit has a molecular mass of 120-155 kDa (35,37,39) and bears the catalytic activity. In the absence of Ca^{2+} the dissociation constant of the calmodulin-catalytic subunit complex is more than 5×10^{-5} M. When calmodulin is saturated with Ca^{2+}, its affinity for the catalytic subunit increases 10,000-fold (dissociation constant 10^{-9} M) and the kinase is maximally activated (35). Smooth muscle MLCK is a highly specific enzyme; it phosphorylates only myosin light chains (35,41). Light chains from striated muscles are phosphorylated with higher K_m and lower V_{max} than smooth muscle

LC (35,37), and isolated LC is phosphorylated more rapidly than LC in myosin filaments (35). On the basis of kinetic data it has been calculated that the rate of LC phosphorylation upon stimulation of smooth muscle is fast enough to account for the observed rate of cross-bridge cycling (calculated from the actomyosin MgATPase activity) and rate of tension development (34,43). This further supports the hypothesis that smooth muscle contraction is regulated by phosphorylation of the LC.

The Ca^{2+}-dependent MLCK can be phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase, which results in a decrease in the rate of LC phosphorylation (38-41,44-46). This decrease is due to the fact that phosphorylation of the catalytic subunit of MLCK decreases its affinity for calmodulin (38-41,45-48). These results suggest that a high cAMP-dependent protein kinase activity in smooth muscle may interfere with contraction. Experiments with crude actomyosin and chemically skinned smooth muscle support the hypothesis. Incubation with cAMP-dependent protein kinase caused a shift to higher $[Ca^{2+}]$ of the curves describing the $[Ca^{2+}]$ dependence of LC phosphorylation, activation of MgATPase activity, actomyosin superprecipitation, and tension development (49-53). The effects of the cAMP-dependent protein kinase could be reversed by adding excess calmodulin (44,48,50,52). Based on the above findings, it has been proposed that the relaxing effect of certain β-adrenergic agonists, such as epinephrine and isoproterenol, on smooth muscle may be mediated by

increased levels of cAMP, which stimulates the phosphorylation and hence inactivation of MLCK (38,41,45,46). However, evidence for such a pathway in smooth muscle is lacking. In fact, it has been reported by Miller et al. (40) that addition of cAMP to tracheal smooth muscle homogenates or relaxation of trachea with isoproterenol had no effect on the MLCK activity. These researchers proposed that cAMP-dependent effects on cytoplasmic Ca^{2+} concentration might be more important than MLCK phosphorylation in mediating relaxation.

D. Myosin light chain phosphatase

Myosin light chain phosphatase has been investigated less extensively than the kinase. A number of laboratories has described, purified, and characterized several smooth muscle phosphatases from gizzard (54-58) and aorta (59,60) that preferentially dephosphorylate the LC. These phosphatases differ in subunit composition and molecular mass, in specificity towards isolated LC, myosin, and other proteins, in divalent cation requirement, and in inhibition by various compounds. Contrary to MLCK, which has a strict substrate specificity, the phosphatases described to date do not appear to be specific for the LC, since they can dephosphorylate other cellular proteins as well. Therefore, it is not clear which, if any, of these enzymes regulate the level of myosin phosphorylation *in vivo*. A role of myosin phosphatase in the regulation of smooth muscle contraction has been implied by experiments that showed inhibition of the actin-activated

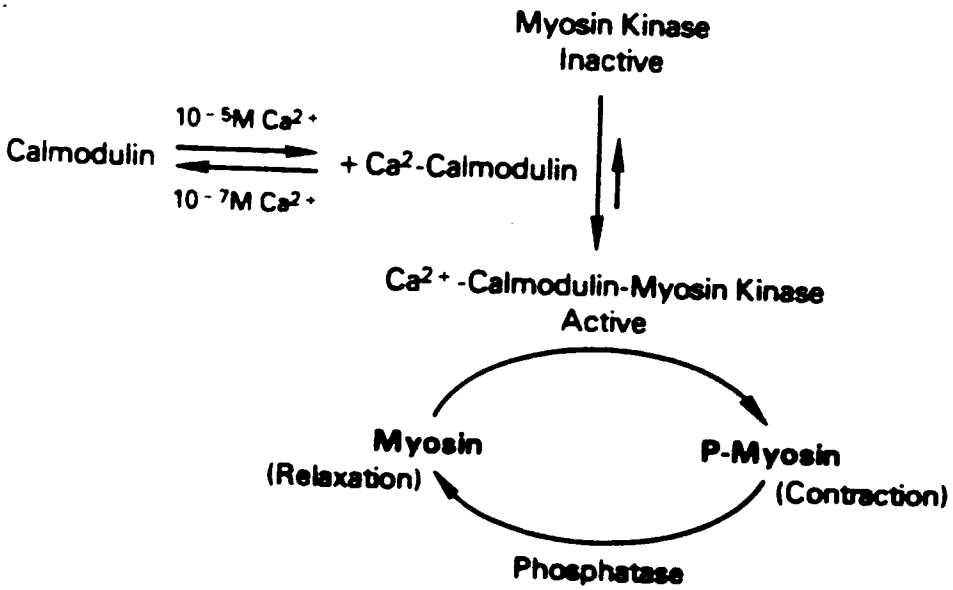
MgATPase activity of phosphorylated myosin (58) and reversal (58) or delay (60) of the superprecipitation of actin and phosphorylated myosin when the phosphatase was added to actomyosin. These effects were always accompanied by LC dephosphorylation.

E. Mechanism of regulation of smooth muscle contraction

The biochemical investigations on the role of LC phosphorylation support the model outlined in Figure 2 for the regulation of smooth muscle contraction. In relaxed muscle with low cytoplasmic Ca^{2+} concentration, little Ca^{2+} is bound to calmodulin. Activation of MLCK is minimal, and the phosphate content of LC is low. Stimulation of the muscle causes a rise in cytoplasmic Ca^{2+} concentration from 10^{-7} M to 10^{-6} - 10^{-5} M. This produces an increase in Ca^{2+}_4.calmodulin, activating MLCK, which phosphorylates the LC. Phosphorylation results in an increase in the actin-activated MgATPase activity of myosin and contraction ensues. When the stimulus no longer exists, Ca^{2+} is sequestered from the cytoplasm and MLCK is inactivated. The level of LC phosphorylation is then lowered by myosin phosphatase, resulting in inactivation of the myosin MgATPase and relaxation.

The question arises: how does the LC regulate the interaction between myosin and actin? To answer this question it is necessary to know the structure and the exact location of the LCs on the smooth muscle myosin molecule. The only information available at present comes from proteolytic digestion studies (16). Controlled proteolysis cleaves the myosin molecule in two parts: heavy

Figure 2. Schematic representation of the regulation of smooth muscle contraction by Ca^{2+}. Reproduced from reference 47.


Myosin Kinase
Inactive
Calmodulin
10 - 5M Ca2 +
10 - 7M Ca2 +
+ Ca2-Calmodulin
Ca2 + -Calmodulin-Myosin Kinase
Active
Myosin
(Relaxation)
P-Myosin
(Contraction)
Phosphatase

meromyosin (HMM), which is the part of the molecule sticking out from the filament surface, and light meromyosin, which belongs to the filament bundle. Heavy meromyosin can be further hydrolyzed to subfragment-1, which is the myosin head, and subfragment-2, which connects each head to the tail of the molecule. Gizzard HMM requires phosphorylation for interaction with actin, but subfragment-1 can interact with actin regardless of LC phosphorylation. These studies suggest that the LC may be located at the neck region of the myosin head and extend across the junction of subfragment-1 and subfragment-2, which is one of the two hinge regions of myosin (the other being the junction of subfragment-2 and light meromyosin). One can further hypothesize that at that position the LC may function by controlling the structure or mobility of the myosin head. During rest the LC may lock the head in a conformation or orientation which is unable to interact with actin. Phosphorylation relieves the inhibitory effect of the LC, possibly by altering its binding to the heavy chain, thus allowing the myosin head to adopt the correct orientation or structure for interaction with actin. Studies with scallop myosin showed that, although the C-terminal region of the regulatory light chain (equivalent to smooth muscle LC) remains stationary, the N-terminal portion can move with respect to the essential light chain (the other class of light chains on the myosin head) (61). It is intriguing to speculate that similar movement is caused in smooth muscle myosin by phosphorylation of the LC, which takes place at the N-terminal region (p. 8).

F. Myosin light chain phosphorylation in intact smooth muscle

Thus far I have reviewed the biochemical mechanisms for Ca^{2+} regulation of smooth muscle actomyosin *in vitro*. Now we must ask: what happens *in vivo*? To examine the regulation of contraction *in vivo*, researchers have chosen the closest approximation, that is the study of the intact muscle after dissection from the animal, which allows direct measurement of the response of the muscle to various treatments. Experiments with carotid artery (62-65), uterus (66-70), and trachea (71-74) showed that stimulation of contraction by a host of agents including K^+, norepinephrine, and carbamylcholine was accompanied by increased LC phosphorylation. Decreases in the extent of LC phoshorylation have been measured during relaxations induced by removal of the stimulus (64,65,74,75) or treatment with a relaxant agent such as EGTA, phenothiazines, and isoproterenol (63,67,68,70,71,76). However, there is no exact temporal relationship between contraction-relaxation and phosphorylation-dephosphorylation. Light chain phosphorylation in response to stimulation usually precedes tension development, reaches a peak before maximal tension is attained, and declines while tension is still developing or has reached a plateau (64-66,72,74). There are also cases in which contraction is not associated with LC phosphorylation. In aorta long preincubation with 5 mM Ca^{2+} resulted in a high level of phosphorylation independent of tension, whereas long preincubation with 5 mM EGTA resulted in low levels of phosphorylation independent of tension

(77). Stretching of carotid arteries to 1.7 times their resting length prevented active tension development (apparently by eliminating the overlap between thick and thin filaments), while the LC was maximally phosphorylated, even in the absence of external stimulus (78). When the stretch was released, tension developed immediately, but the LC was dephosphorylated (79).

In conclusion, the experiments described above support the hypothesis that LC phosphorylation regulates smooth muscle contraction, but also show that phosphorylation alone cannot explain the complex behavior of smooth muscles. Apparently other regulatory mechanisms operate as well. In this context, it is proper to remember the actin filament-mediated mechanisms mentioned previously (pp. 4-5).

G. Multiple phosphorylation of myosin light chain

Until recently the LC was thought to have only one phosphorylation site and, therefore, incorporate a maximum of 1 mol phosphate/mol. In 1982 it was reported that the Ca^{2+}-activated phospholipid-dependent protein kinase or protein kinase C can phosphorylate gizzard LC, both in isolated form and in whole myosin (80). Protein kinase C phosphorylates a different site on the LC than does MLCK, and sequential phosphorylation of HMM (myosin without its hydrophobic tail, preferred for its solubility) by MLCK and protein kinase C results in diphosphorylation of the LC (81). The site of phosphorylation by protein kinase C was identified as a threonine residue (82). Phosphorylation of HMM by either one of the

two kinases decreases the affinity of the other kinase for HMM (82). The two kinases have opposite effects on the MgATPase activity of HMM. Whereas phosphorylation of unphosphorylated HMM by MLCK increases its actin-activated MgATPase 40-fold, subsequent phosphorylation of this phosphorylated HMM by protein kinase C decreases its actin-activated MgATPase 2-fold (81).

Following the first demonstration of LC diphosphorylation, a gizzard myosin preparation was described which, when incubated with ATP, gave rise to diphosphorylated as well as monophosphorylated LC (83). The endogenous kinase responsible for the observed diphosphorylation was not identified. However, diphosphorylated LC could also be obtained by prolonged incubation of isolated LC with high amounts of MLCK. Monophosphorylated LC contained phosphoserine (Ser-P), while diphosphorylated LC contained Ser-P and phosphothreonine (Thr-P) (83).

Two more laboratories have reported diphosphorylation of gizzard LC to date (84,85). Both reported that intact myosin could incorporate up to 2 mol phosphate/mol LC when incubated with high amounts of MLCK. Contrary to phosphorylation by protein kinase C, phosphorylation of the second site by MLCK markedly increases the actin-activated MgATPase activity of myosin (84,85). The second phosphorylation site is a threonine residue (85,86), but it is distinct from the threonine residue phosphorylated by protein kinase C, since sequential phosphorylation of myosin with protein kinase C and large amounts of MLCK results in the incorporation of 3 mol

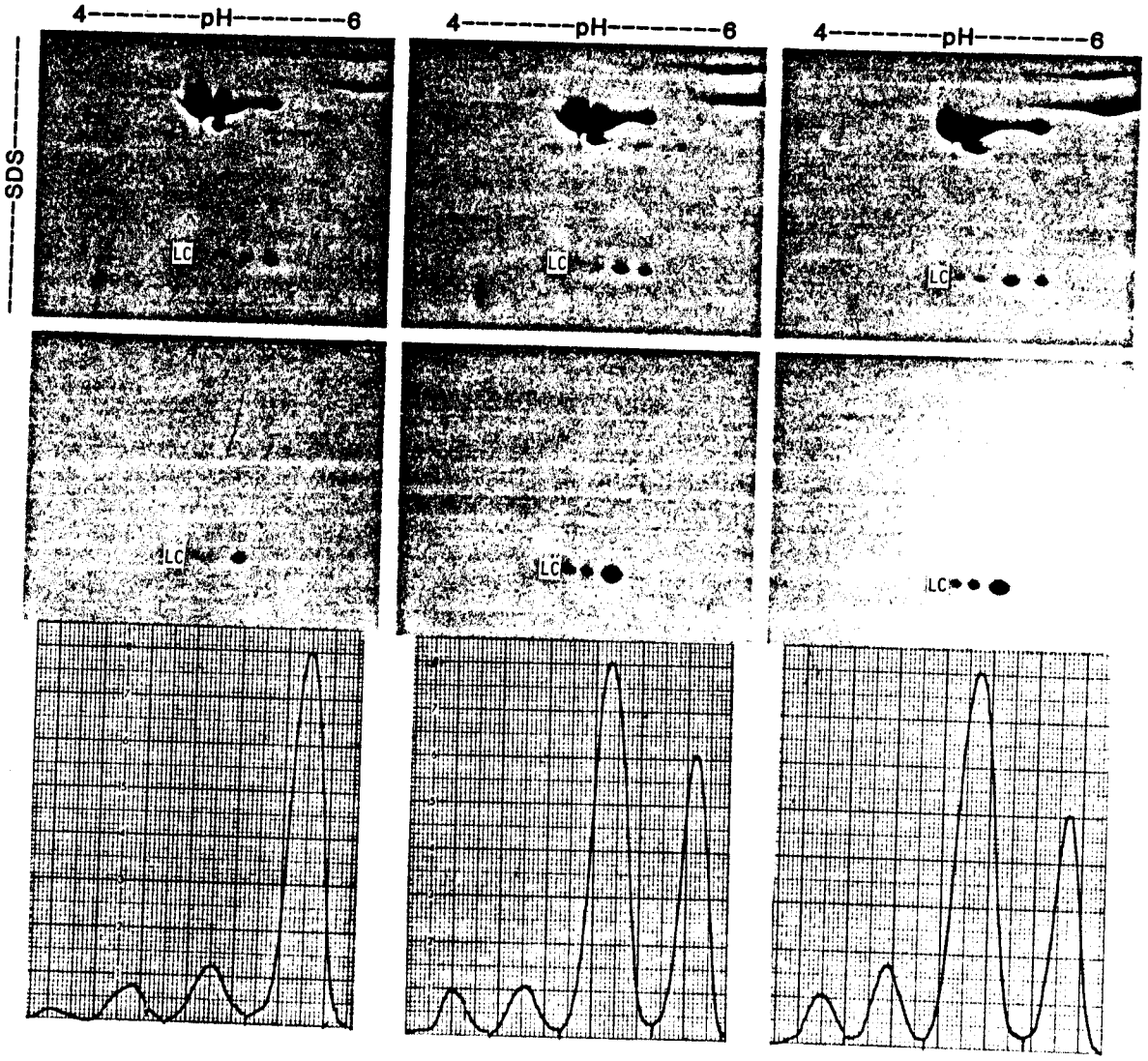
phosphate/mol LC (87). Following chymotryptic peptide separation, amino acid analyses, and partial sequence determinations, the second site of phosphorylation by MLCK was shown to be threonine 18, next to the first phosphorylation site (serine 19), whereas the site of phosphorylation by protein kinase C is threonine 9 or 10. It is remarkable that all three phosphorylation sites are located within only 10 or 11 amino acids at the N-terminal region of the LC.

H. Multiple forms of the 20-kDa myosin light chain

The relationship between LC phosphorylation and contraction at the level of intact smooth muscle has been studied in either of two ways. The tissue is incubated with $^{32}P_i$, which permeates the plasma membrane and labels the intracellular ATP pool through oxidative phosphorylation and substrate level phosphorylation. The [γ-^{32}P]ATP is then used by the kinase to label the LC. The muscle is quick-frozen at various functional states and LC phosphorylation is determined by separating the LC from the other ^{32}P-labeled proteins (usually by electrophoresis) and by measuring its radioactivity. Alternatively, the tissue is frozen without radioactive labeling and phosphorylation is determined by quantitating phosphorylated and unphosphorylated LC. This requires a system that will separate the two forms from each other and from all the other proteins of the tissue. Two-dimensional gel electrophoresis has been favored by most investigators. When this method was used with arterial or uterine proteins, the LC was resolved not only in its phosphorylated and unphosphorylated form,

but in two additional forms of lower isoelectric point (pI) (64,69,70,78,79,88, and Figure 3). The nature of these spots has been the subject of controversy. It has been argued that they originate from the two main LC spots as the result of artifactual charge modification (64,69), but data have also been presented against this hypothesis (88). I began my research with the effort to clarify this controversy.

Figure 3. Two-dimensional polyacrylamide gel electrophoresis of proteins from ^{32}P-labeled porcine carotid arteries frozen at resting tension (left; illustration is rotated by 90°), after stimulation with 100 mM KCl (middle), or after stretching to 1.7 times the resting length (right). Top: Coomassie blue-stained gels. Middle: autoradiograms of the gels. Bottom: densitometric tracings of the LC spots in the Coomassie blue-stained gels. Reproduced from reference 79.


4--------pH--------6
4--------pH--------6
4--------pH--------6
SDS
LC
LC
LC
LC
LC
LC

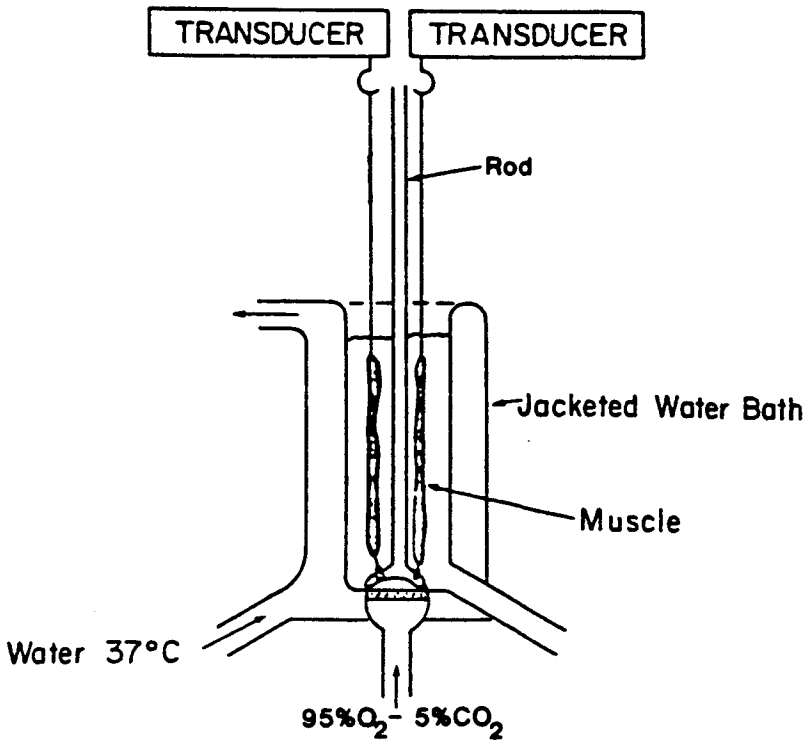
III. EXPERIMENTAL PROCEDURES

Two porcine smooth muscles were used throughout this work: carotid artery and aorta. Carotid artery is ideal for the study of the contractile response of smooth muscle to various treatments and the relationship between contraction and LC phosphorylation. Aorta, on the other hand, is less responsive to stimulation, but it is more massive than the two carotid arteries and is therefore more suitable for preparative work. The two tissues have the same LC patterns.

A. Preparation of ^{32}P-labeled proteins from carotid arteries

^{32}P-labeled proteins from porcine carotid arteries were prepared as described (79). Carotid arteries were obtained from freshly slaughtered hogs at a local abattoir (American Meat Packing Corp., Chicago) and were transported to the laboratory in ice-cold physiological salt solution (PSS) containing 130 mM NaCl, 4.7 mM KCl, 1.2 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. The arteries were cleaned of surrounding fat and adventitia. Segments of arteries were cut helically (according to the orientation of the smooth muscle cells) to produce strips approximately 5 cm long and 0.5 cm wide. The strips were tied at each end with surgical suture and were mounted in a jacketed incubation chamber (Figure 4) with one end attached to a stationary hook and the other end to a force-displacement transducer. Tension was recorded with a Grass polygraph. The

Figure 4. Muscle incubation chamber. Depicted from reference 89.


TRANSDUCER
TRANSDUCER
Rod
Jacketed Water Bath
Muscle
Water 37°C
95%O2- 5%CO2

muscle strips (two per chamber) were incubated in PSS aerated with a mixture of 95% O_2 and 5% CO_2 at 37°C, while a resting (passive) tension was applied to them to simulate a mean arterial blood pressure of 100 mm Hg (90). The resting tension was calculated for each strip according to the equation

$$\text{Tension (g-wt)} = 10.8 \frac{\text{g-wt}}{\text{cm}^2} \times \text{length (cm)} \times \text{width (cm)} \quad \text{(Equation 1)}$$

which was derived from the law of Laplace. After 10 min the PSS was replaced by 60 ml of PSS containing 2 mCi of carrier-free $^{32}P_i$ from Amersham. After 1 h of incubation the extracellular ^{32}P was removed by extensive washing of the strips (15 washes over a period of 30 min). Then the muscles were treated in either of the following ways:

1. Muscles were frozen at resting tension by immersion in liquid nitrogen.

2. Muscles were stimulated by 100 mM KCl in isotonic salt solution. The high K^+ concentration depolarizes the plasma membrane and allows influx of Ca^{2+} through voltage-sensitive channels. The strips were frozen with liquid nitrogen after 1 min, at which time they had developed an average active tension of 40 g-weight.

3. Muscles were stimulated by 50 μM norepinephrine (natural neurotransmitter) in PSS. The strips were frozen after 2 min, at which time they had developed an average active tension of 20 g-weight.

4. Strips were stretched to 1.7 times their length at resting tension and were frozen immediately.

5. Strips were stretched to 1.7 times their resting length and were stimulated by 100 mM KCl in isotonic salt solution. No active tension developed. The strips were frozen after 1 min.

Each frozen strip was pulverized to a powder by percussion using liquid nitrogen-chilled steel mortars and pestles. The powder was mixed thoroughly with 10 ml of 3% perchloric acid and was centrifuged at 27,000 x g for 15 min. The supernatant, containing soluble cellular metabolites, was saved for determination of the specific radioactivity of [^{32}P]phosphocreatine as reference for the calculation of the [^{32}P]phosphate content of the LC (section C). The pellet was used for protein preparation. It was washed twice by suspension in 30 ml of 2% trichloroacetic acid (TCA), 5 mM KH_2PO_4 and centrifugation at 27,000 x g for 10 min. Then the proteins were solubilized by addition of 0.5 ml of saturated Na_2HPO_4 solution at 37°C (approximately 3 M), 0.15 ml of 3% sodium dodecyl sulfate (SDS), and 0.35 ml of water, and by homogenization with a Brinkmann Polytron at medium speed for 1.5 min. The resulting suspension was dialyzed against 20 l of 0.02% SDS at room temperature overnight. The contents of the dialysis bag were then centrifuged at 78,000 x g for 30 min. The supernatant, containing solubilized proteins, was saved and the pellet was discarded. Protein concentration was determined by means of the biuret reaction (91), measuring the absorbance at 320 nm (92). Appropriate aliquots were lyophilized for electrophoresis.

B. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis, consisting of isoelectric focusing (IEF) in the first dimension and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, was performed according to O'Farrell (93). The IEF gels (2.5 mm in diameter) contained 3.78% acrylamide, 0.22% bisacrylamide, 8.5 M urea, 2% Nonidet P40, 2% ampholytes, pH 4-6 (Bio-Rad), 0.01% ammonium persulfate, and 0.1% N,N,N',N'-tetramethylethylene-diamine. The gels were pre-electrophoresed at 200 V for 20 min and at 400 V for 20 min. The cathode solution was 20 mM NaOH and the anode solution was 10 mM H_3PO_4. The lyophilized proteins were dissolved in IEF sample solution, containing 9 M urea, 2% Nonidet P40, 2% ampholytes, and 3% dithiothreitol (DTT), and 400-450 µg of proteins were loaded on each gel. Electrophoresis was carried out at 400 V overnight (16 h) and at 1000 V for 1 h. Each IEF gel was then shaken in 4 ml of SDS sample buffer, containing 10% glycerol, 0.5% DTT, 2.3% SDS, 0.063 M Tris.HCl, pH 6.8, and 0.001% bromphenol blue, for 20-60 min. After this equilibration the IEF gel was applied onto a slab gel for SDS-PAGE. The slab gels (1.6-mm thick) consisted of a stacking gel on top of a resolving gel. The composition of the resolving gel was 14.6% acrylamide, 0.4% bisacrylamide, 0.1% SDS, 0.375 M Tris.HCl, pH 8.8, 0.035% ammonium persulfate, 0.05% N,N,N',N'-tetramethylethylene-diamine. The composition of the stacking gel was 2.92% acrylamide, 0.08% bisacrylamide, 0.1% SDS, 0.125 M Tris.HCl, pH

6.8, 0.04% ammonium persulfate, 0.1% N,N,N',N'-tetramethylethylenediamine. The electrophoresis buffer was 0.025 M Tris base, 0.192 M glycine, and 0.1% SDS. Electrophoresis in the second dimension was performed at 130 V for 1 h and at 170 V until the bromphenol blue tracking dye left the bottom of the gels. The gels were stained in 0.12% Coomassie blue, 50% methanol, 5% acetic acid for 3 h, destained in 40% methanol, 5% acetic acid, and stored in 7% acetic acid. This staining procedure was also used for all SDS gels described below. The distribution of staining intensity among the LC spots was determined with a Helena scanning densitometer equipped with automatic integrator. Certain gels were dried and subjected to autoradiography with Kodak X-Omat AR films and Du Pont Cronex Lightning-Plus intensifying screens.

C. Quantitation of [^{32}P]phosphate incorporation into the myosin light chain of carotid arteries

The two-dimensional polyacrylamide gels described above were used to calculate the mol of [^{32}P]phosphate incorporated per mol of LC, as well as the mol of [^{32}P]phosphate per mol of each LC spot. For this purpose the four LC spots were excised from the gels and were incubated with 0.5 ml of 30% H_2O_2 at 110°C in capped scintillation vials until the gel pieces were dissolved. The radioactivity of each spot was then determined by liquid scintillation counting.

To convert the radioactivity of each LC spot to mol of [^{32}P]phosphate, it is necessary to know the specific radioactivity

of the γ-phosphate group of ATP, which is transferred to the LC through the action of the kinase. In muscle the enzyme creatine kinase catalyzes the rapid exchange of phosphate groups between two high-energy compounds, phosphocreatine and ATP. The creatine kinase reaction for ^{32}P-labeled muscle is :

$$[\gamma\text{-}^{32}P]ATP + \text{creatine} \rightleftharpoons ADP + [^{32}P]\text{phosphocreatine}$$

It has been shown that at the end of the 1 h incubation of carotid arteries with $^{32}P_i$ the specific activities of $[^{32}P]P_i$, $[\gamma\text{-}^{32}P]ATP$, and $[^{32}P]$phosphocreatine are identical (62). This allows the replacement of the time-consuming determination of the specific activity of $[\gamma\text{-}^{32}P]ATP$ by that of $[^{32}P]$phosphocreatine and $[^{32}P]P_i$. The assay is based on the fact that phosphocreatine is more susceptible to acid hydrolysis than other phosphocompounds (e.g. sugar phosphates), which have slower turnover and therefore lower specific radioactivity. The $[^{32}P]P_i$ liberated by the hydrolysis (and that already present in the muscle extract) reacts with molybdate under strongly acidic conditions according to the reaction

$$H_3PO_4 + 12\ H_2MoO_4 \rightleftharpoons H_3PMo_{12}O_{40} + 12\ H_2O$$

The product of the reaction, dodecamolybdatophosphoric acid, can be extracted selectively with isopropyl acetate.

The assay was performed as follows (94): One ml of the supernatant after centrifugation of the pulverized muscle (p. 27) was mixed thoroughly with 1.0 ml of water, 0.4 ml of 5 M $HClO_4$,

and 1.6 ml of 0.05 M Na_2MoO_4. The mixture was incubated at 37°C for 40 min and was mixed vigorously with 4.0 ml of isopropyl acetate. After separation of the phases the absorbance of the upper (organic) phase at 310 nm was measured and was divided by the extinction coefficient of dodecamolybdatophosphoric acid (23.8 $mM^{-1}cm^{-1}$) to give the phosphate concentration. The radioactivity of the upper phase was also determined. From the two values the specific radioactivity of [^{32}P]phosphocreatine was obtained (usually 10-20 cpm/pmol). By dividing the cpm of each LC spot by the specific radioactivity of [^{32}P]phosphocreatine, the pmol of [^{32}P]phosphate in each spot were determined.

The pmol of LC in each gel were determined as follows: Myosin comprises 8.5% of the porcine carotid artery proteins (95); therefore the µg of myosin in each gel were calculated by multiplying the µg of protein loaded on the IEF gel by 0.085. From the µg of myosin the pmol of LC were calculated by assuming a molecular mass of 500 kDa for myosin and 2 mol LC/mol myosin. By dividing the pmol of [^{32}P]phosphate in all four LC spots by the pmol of LC, the mol of [^{32}P]phosphate per mol of LC were calculated. To determine the incorporation of [^{32}P]phosphate into each spot, the pmol of protein in each LC spot were calculated from the pmol of total LC and from the percent staining intensity of each spot, assuming that the staining intensity is proportional to the amount of protein. By dividing the pmol of [^{32}P]phosphate in each

spot by the pmol of LC in that spot, the mol of [^{32}P]phophate per mol of LC spot were calculated.

D. Dephosphorylation of myosin

Arterial smooth muscle myosin was dephosphorylated either in the intact tissue or in tissue homogenates. In the first case helical strips of carotid arteries were stored in glucose-free PSS at 4°C for 3 days. Then they were frozen, pulverized, solubilized, and analyzed by two-dimensional gel electrophoresis as described in section A. In the second case porcine carotid arteries and aortas were transported from the slaughterhouse to the laboratory in ice-cold 150 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid, pH 7.3. Each tissue was cleaned from surrounding fat and adventitia and was transferred into an ice-jacketed Waring blender with 30 volumes of the above buffer containing in addition 1 mM EGTA and 1 mM DTT. The tissue was homogenized for 4 min with a 15-s interval every 1 min, and was stirred at room temperature for 2 h. The homogenate was then made 5% in TCA and was centrifuged at 27,000 x g for 15 min. The supernatant was discarded and the pellet was washed, solubilized, and analyzed by two-dimensional gel electrophoresis as described in section A.

E. Preparation of crude aorta actomyosin

Crude actomyosin was prepared by modification of a published procedure (96). All operations were performed at 0-4°C. Aortas were obtained from freshly slaughtered hogs and were transported to the laboratory in isotonic saline containing 150 mM NaCl, 25 mM

histidine.HCl, and 0.15 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.0. Surrounding fat was removed and the adventitia and intima were peeled off with forceps, exposing the media layer. Two hundred g of the media layer were minced with an electric mincer and were washed with 800 ml of isotonic saline three times. The washed mince was mixed with 500 ml of 20 mM KCl, 20 mM histidine.HCl, 1 mM $MgSO_4$, 1 mM DTT, 0.15 mM PMSF, 1 mg/l leupeptin, 2 mg/l soybean trypsin inhibitor, and 70 µM streptomycin sulfate, pH 6.8, and was homogenized with the Polytron at full power for 1.5 min with a 2-min interval every 0.5 min. The homogenate was centrifuged at 10,000 x g for 15 min and the supernatant, containing soluble cytoplasmic proteins, was discarded. The pellet was suspended in 250 ml of 0.6 M KCl, 20 mM histidine.HCl, 2 mM EGTA, 1 mM EDTA, 10 mM ATP, 1 mM DTT, 0.15 mM PMSF, 1 mg/l leupeptin, 2 mg/l soybean trypsin inhibitor, and 70 µM streptomycin sulfate, pH 7.8, and was homogenized with the Polytron at full power for 10 s. The resulting homogenate was centrifuged at 22,000 x g for 15 min and the supernatant, containing extracted myosin and actin, was subjected to another centrifugation at 100,000 x g for 1 h, in order to precipitate membranes and filamentous actin. The supernatant of the last centrifugation was dialyzed against 28 l of 5 mM potassium phosphate, pH 7.0, overnight. The pellets of the last two centrifugations were discarded.

The following day the contents of the dialysis bags were centrifuged at 10,000 x g for 15 min. The supernatant, containing

proteins soluble at low salt concentration, was discarded. The soft white pellet, consisting mainly of actin and myosin, was suspended in 100 ml of 10 mM KCl with a Potter-Elvehjem homogenizer and was recentrifuged. This washing procedure was repeated once. The final crude actomyosin pellet was used for various experiments immediately.

F. Preparation of myosin light chains

Light chains were prepared from either unphosphorylated or phosphorylated crude actomyosin as described (97).

1. Unphosphorylated myosin light chains were isolated for subsequent purification of LC forms. The crude actomyosin prepared as described above was mixed with solid guanidine.HCl, DTT, and water to a final volume of 100 ml and concentration of 5 M guanidine.HCl, 2.5 mM DTT. The proteins were dissolved with a Potter-Elvehjem homogenizer and the solution was stirred at room temperature for 1 h to allow complete denaturation of myosin. The solution was then diluted slowly and with stirring, first with 100 ml of cold water and then with 400 ml of cold ethanol. After 30 min of additional stirring the suspension was centrifuged at 10,000 x g for 15 min. The pellet, containing mainly myosin heavy chains, actin, and tropomyosin, was discarded. The supernatant contained mainly the two classes of light chains (20- and 17-kDa), actin, and tropomyosin. Further enrichment of the supernatant in light chains was obtained by overnight storage in the refrigerator, upon which more actin and tropomyosin precipitated, whereas the light chains remained in solution. After a new centrifugation the supernatant

was dialyzed against 28 l of 5 mM potassium phosphate, pH 7.0, in the coldroom overnight. The light chains precipitated and were collected by centrifugation at 27,000 x g for 15 min. The pellet was solubilized with Na_2HPO_4 and SDS as described on p. 27.

2. Phosphorylated myosin light chains were isolated for two-dimensional IEF, electrophoresis with sodium thioglycolate, and pI determination of the LC spots. One-tenth portions (by weight) of the crude actomyosin prepared in section E were suspended in incubation buffer consisting of 35 mM KCl, 50 mM 3-(4-morpholino)propanesulfonic acid, 1 mM DTT, 0.15 mM PMSF, 1 mg/l leupeptin, and 2 mg/l soybean trypsin inhibitor, pH 7.0, to a total volume of 10 ml. The suspension was incubated at 25°C with stirring. Phosphorylation was initiated by adding 52 µl of 1 M $MgSO_4$, 104 µl of 10 mM $CaCl_2$, and 208 µl of 50 mM ATP (final concentrations 5, 0.1, and 1 mM, respectively), and was terminated after various periods of time by adding 7.5 g of guanidine.HCl (final volume 15.7 ml and guanidine.HCl concentration 5 M). Light chains were then isolated by ethanol fractionation as described above.

G. Purification of 20-kDa light chain forms

A 3-mm thick preparative SDS-polyacrylamide gel was cast having the same composition as the gels used for two-dimensional gel electrophoresis (pp. 28-29) with the exception that the stacking gel was 5% instead of 3% polyacrylamide. The higher polyacrylamide content produced a better sample well, which was formed by inserting a 10-cm wide comb with no indentations at the top of the stacking

gel immediately after pouring it. Five mg of lyophilized, unphosphorylated myosin light chains were dissolved in 1 ml of SDS sample buffer (p. 28) and were heated in boiling water bath for 1.5 min. The solution was layered carefully with a syringe at the bottom of the sample well under electrophoresis buffer, and electrophoresis was started. During SDS-PAGE a preparative IEF gel of the same dimensions as the preparative SDS gel was cast. The composition of the gel was that of the cylindrical IEF gels (p. 28), except that the polyacrylamide content was 5% instead of 4% (for greater mechanical strength) and the ampholytes were 20-fold diluted Pharmalyte, pH 4.5-5.4 (for higher resolution). After electrophoresis the SDS gel was stained until the LC band became visible (5-10 min). After destaining for an equal time the LC band was excised in one piece and was shaken first in 10 ml of water for 30 min and then in 10 ml of IEF sample solution (p. 28) for 1 h. After this equilibration the LC band was applied to the top of the preparative IEF gel (between the glass plates) and was sealed with hot 1% agarose. The gel was electrophoresed at 150 V for 16 h. For location of the bands of the LC forms, two vertical guide strips were excised from the gel for staining. The guide strips were first shaken in 10% TCA for 5 h or more and in 30% methanol, 10% acetic acid for 1 h. Then they were stained in 0.02% Coomassie blue, 30% methanol, 10% acetic acid for 5 h and destained in 30% methanol, 10% acetic acid. This staining procedure was also used for all IEF gels described below. The strips were then aligned

with the unstained gel and the bands containing the two unphosphorylated LC forms were excised and cut in 2-mm wide pieces. To extract the protein, the gel pieces of each LC form were shaken in 10 ml of 0.5% SDS, 0.1 M sodium phosphate, 1 mM EDTA, pH 7.0, for one day. The extract was decanted into a centrifuge tube and the extraction was repeated. The combined extracts were centrifuged at 27,000 x g for 15 min to remove gel pieces. The supernatant was dialyzed against 28 l of distilled water at room temperature for two days. The retentate was centrifuged at 27,000 x g for 15 min and the supernatant, containing purified LC, was lyophilized. The pellet, containing gel material, was discarded.

H. Two-dimensional isoelectric focusing

Eighty µg of myosin light chains from crude aorta actomyosin phosphorylated for 5 or 15 min were analyzed by IEF at pH 4.5-5.4 in a cylindrical gel (1.8 mm in diameter). After electrophoresis the gel was shaken in 5 ml of water for 10 min. Then it was applied to the top of a 1.6-mm thick slab IEF gel of the same composition as the preparative IEF gel described above. Electrophoresis in the second dimension was performed at 200 V for 8 h. The gel was stained as described before.

I. Determination of isoelectric points of the light chain forms

Isoelectric points were determined with the help of the following marker proteins from Sigma: soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A from milk (pI 5.13), and carbonic anhydrase B from bovine erythrocytes (pI 5.65). Seventy to ninety µg of myosin

light chains from crude aorta actomyosin phosphorylated for 1, 5, or 30 min and 2.5-5 µg of isoelectric point markers were subjected to IEF at pH 4-6. The IEF gels were stained as described before.

J. Peptide mapping

One hundred µg of each purified LC form (section G) were dissolved in 0.5 ml of 50 mM NH_4HCO_3 and were hydrolyzed with 1 µg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) at 37°C for 6 h (98). The hydrolyzate was lyophilized and redissolved in 20 µl of 2% NH_4OH. Twenty to fifty µg of hydrolyzed protein were spotted on a 20 x 20-cm Kodak Chromagram 13255 cellulose sheet. The sample was electrophoresed in pyridine:glacial acetic acid:water (1:10:189), pH 3.5, in a Gelman Deluxe electrophoresis chamber at 300 V for 1.5 h. The sheet was then dried under a stream of cold air and was chromatographed in a 27 x 7 x 21-cm Brinkmann glass tank in 1-butanol:pyridine:glacial acetic acid:water (15:10:3:12), in a direction perpendicular to that of electrophoresis. After the chromatography solvent reached the top, the sheet was dried in an oven at 80°C, sprayed with 0.2% ninhydrin in acetone, and returned to the oven for color development (10-15 min).

K. Phosphorylation of aorta homogenate and actomyosin by endogenous kinases

1. Two g of fresh porcine aortic media (p. 33) were chopped with scissors and were mixed with 10 ml of ice-cold incubation buffer (p. 35). The tissue was disrupted with two 15-s pulses of

the Polytron at full power. The homogenization was performed on ice and the homogenate was allowed to cool for 2 min between the pulses. The volume was brought to 24 ml with incubation buffer and the homogenate was stirred at 25°C. Phosphorylation was initiated by the addition of $MgSO_4$, $CaCl_2$, and ATP to final concentrations of 5, 0.1, and 1 mM, respectively. At varying times of incubation aliquots were removed and were made 5% in TCA. After centrifugation at 27,000 x g for 15 min the pellets were washed and solubilized for two-dimensional gel electrophoresis as described in sections A and B. Approximately 400 µg of proteins were applied to each gel.

2. Crude actomyosin was phosphorylated as described in section F.2, except that the phosphorylation was not terminated with guanidine.HCl but with TCA as described above for the tissue homogenate. The TCA pellets were treated likewise. Approximately 200 µg of proteins were applied to each gel.

L. ^{32}P-Labeling of aorta actomyosin and quantitation of [^{32}P]phosphate incorporation into the myosin light chain

Crude aorta actomyosin was phosphorylated for 10 or 15 min as described above, with the exception that [γ-^{32}P]ATP was substituted for unlabeled ATP. Carrier-free γ-^{32}P-ATP was prepared by incubating $^{32}P_i$ with GammaPrep-A (Promega Biotec, Madison, WI). The procedure utilizes the substrate level phosphorylation of ADP during the enzymatic conversion of L-α-glycerolphosphate to 3-phosphoglycerate (99). GammaPrep-A

contains all the enzymes and reagents necessary for this pathway, except phosphate. Two mCi of carrier-free $^{32}P_i$ in 50 µl of 10 mM HCl were mixed with the 50 µl of one vial of GammaPrep-A and the solution was incubated at room temperature for 1 h. The reaction was terminated by heating at 90°C for 5 min. To determine the incorporation of ^{32}P into ATP, a minute amount (less than 0.1 µl) of the reaction mixture was spotted on a Brinkmann MN-Polygram Cel 300 PEI polyethyleneimine-cellulose sheet along with a mixture of unlabeled ATP, ADP, and AMP (10 nmol each). The sheet was developed with 1.0 M LiCl (100). After drying, unlabeled nucleotide markers were visualized under ultraviolet light. The sheet was then autoradiographed. The spots corresponding to $^{32}P_i$ and γ-^{32}P-ATP were excised and their radioactivity was determined by liquid scintillation counting. Aliquots of the reaction mixture were diluted with a solution of 50 mM ATP in 10 mM HCl to give a specific radioactivity of 70-100 cpm/pmol [γ-^{32}P]ATP. The analytical concentration of ATP was determined by reading the absorbance at 257 nm (extinction coefficient of ATP at pH 2, 14.6 $mM^{-1}cm^{-1}$, reference 101).

The incorporation of [^{32}P]phosphate into the LC was determined with two-dimensional polyacrylamide gels in a way similar to that described in section C. The radioactivity of each LC spot was divided by the specific radioactivity of the [γ-^{32}P]ATP used for the phosphorylation to give pmol of [^{32}P]phosphate. The pmol of protein in each LC spot were calculated from the µg of proteins

applied to each gel, from the LC content of each sample, and from the distribution of staining intensity among the spots. The LC content of the actomyosin preparations was determined by electrophoresis in 1.6-mm thick SDS-polyacrylamide gels of the same composition as the preparative gel described in section G, except that the resolving gel contained only 7.5% polyacrylamide. Fifteen to twenty-five µg of proteins were applied to 5-mm wide wells at the top of the stacking gel. After electrophoresis, staining, and destaining, each sample lane was scanned and the staining intensity of MHC was expressed as percentage of the total staining intensity. This was taken as the MHC content of the actomyosin sample, assuming that the staining intensity per unit of mass is the same for all proteins. Since one 20-kDa light chain corresponds to one 200-kDa heavy chain in the myosin molecule, the LC content is 1/10 of the MHC content.

By dividing the pmol of [^{32}P]phosphate in each LC spot by the pmol of protein in that spot, the mol of [^{32}P]phosphate per mol of LC spot were obtained.

M. Identification of phosphoamino acids in myosin light chain

The phosphorylated amino acids of total LC as well as of individual LC spots were determined with ^{32}P-labeled carotid arteries and aorta actomyosin (sections A and L), based on a published procedure (102). ^{32}P-Labeled proteins were analyzed by two-dimensional gel electrophoresis (section B) and by SDS-PAGE in 1.6-mm thick gels of the same composition as the preparative SDS gel

described in section G. The amount of proteins applied to the 5-mm wide wells of the stacking gel was 120-250 μg for carotid arteries and 40-80 μg for aorta actomyosin. After electrophoresis the one- and two-dimensional gels were dried without staining. The total LC and the individual radioactive LC spots were located by autoradiography and were excised from the dry gels. Each gel fragment was swollen in 1 ml of 0.1% SDS, 0.5% DTT and was crushed with a glass rod. The LC was extracted by overnight incubation at room temperature with stirring. The extract was removed and the extraction was repeated with 1 ml of 0.1% SDS for 2 h. The combined extracts were centrifuged at 8,000 x g for 10 min to remove remaining gel fragments. The supernatant was made 20% in TCA and was kept at 0°C for 4 h. After centrifugation at 8,000 x g for 15 min the supernatant was discarded, whereas the barely visible pellet was suspended in 0.6 ml of 6 N HCl and was transferred into a 13 x 100-mm screw cap Pyrex tube. The tube was capped tightly and was incubated at 110°C for 2 h. The hydrolyzate was then evaporated in a rotary evaporator with a 50-60°C water bath and the dry residue was dissolved in 10 μl of water. One μg of each Ser-P, Thr-P, and phosphotyrosine (Tyr-P) were added as internal markers and the sample was spotted on a Kodak Chromagram 13255 cellulose sheet. The sheet was electrophoresed with the sample at the cathode in 88% formic acid:glacial acetic acid:water (25:78:897), pH 1.9, or pyridine:glacial acetic acid:water (1:10:189), pH 3.5, at 400 V for 2 h. The sheet was dried and

sprayed with ninhydrin as described in section J, in order to locate the unlabeled phosphoamino acid markers. The sheet was then autoradiographed (usually for one week), in order to locate the [^{32}P]phosphoamino acids of the LC.

IV. RESULTS

A. Are the multiple forms of arterial 20-kDa myosin light chain experimental artifacts?

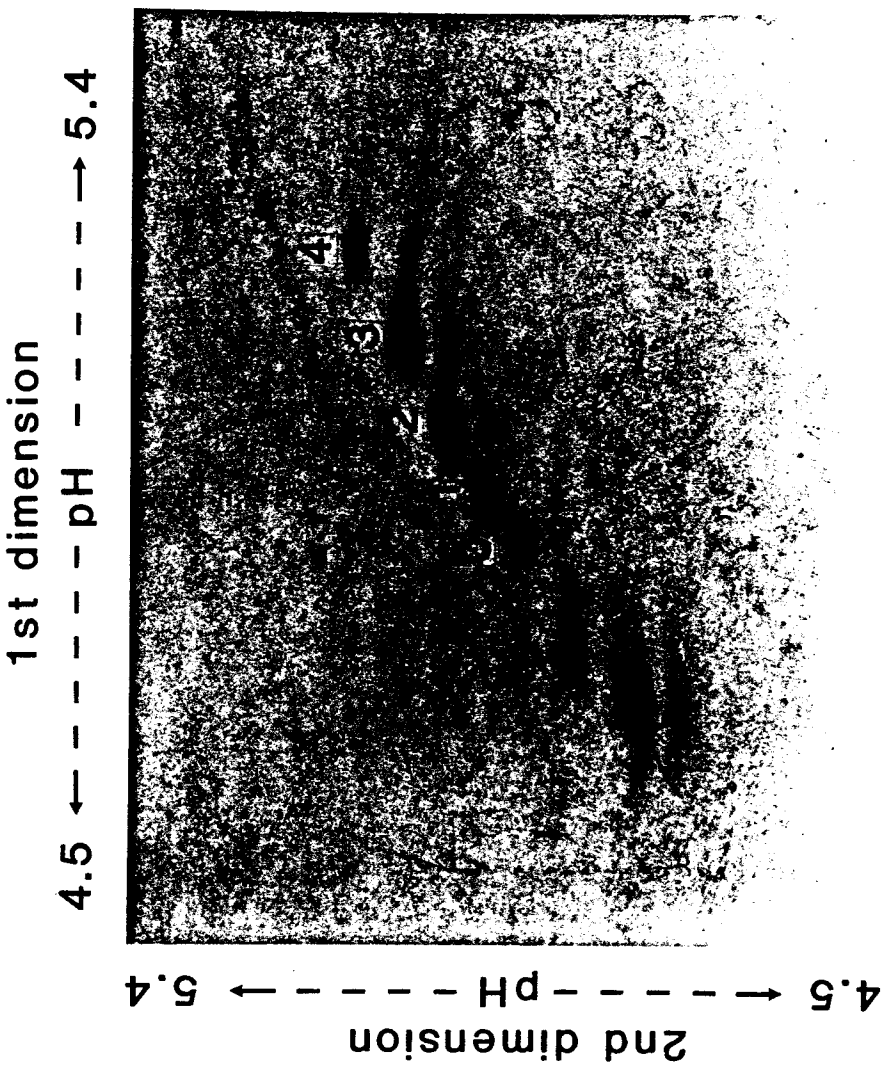
When the proteins of arterial and uterine smooth muscle were analyzed by two-dimensional gel electrophoresis in order to separate and quantitate the unphosphorylated and phosphorylated LC, four closely spaced spots were found at the 20-kDa level (64,69,70,78,79,88). These spots have been numbered 1 through 4 in order of increasing pI in this laboratory. Determining the nature of the four spots is necessary for accurate quantitation of LC phosphorylation in non-radioactive experiments. One explanation for multiple isoelectric variants in a two-dimensional gel is artifactural charge modification due to carbamylation or oxidation (69,93). I examined this possibility in two ways. Gagelmann et al. (88) separated the proteins of carotid arteries by IEF in a cyclindrical gel and applied it on a slab gel for a second IEF. Since proteins are separated by the same principle in both dimensions, they are arranged diagonally in the final two-dimensional gel. However, if any LC spots are produced artifactually during IEF, they should be produced during the second dimension of IEF as well and should appear as additional, off-diagonal spots. The data of Gagelmann et al. showed that all LC spots refocused as discrete and separate proteins and no additional

spots were generated. They concluded from this that the observed LC spots were not the result of charge modification during IEF.

I repeated their experiment, but with a different preparation. Arterial smooth muscle contains large amounts of tropomyosin, whose pI is very similar to that of LC (see, for example, Figure 3 of this text and Figure 1 of reference 88). Because of this, tropomyosin will migrate with the LC in both directions of the two-dimensional IEF system and will obscure its pattern. To circumvent this problem, instead of total arterial proteins I used isolated myosin light chains, which contain only traces of tropomyosin. Since some LC spots disappear due to dephosphorylation (as will be discussed later) during the long preparation, I phosphorylated the crude aorta actomyosin with 1 mM ATP for 5 or 15 min before light chain fractionation (section III.F.2). Surprisingly, this phosphorylation gave rise to a fifth spot, more acidic than spot 1. In accordance with the numbering of the already existing spots, the fifth spot will be referred to as spot 0. By subjecting the phosphorylated myosin light chains to two-dimensional IEF, I confirmed the conclusion of Gagelmann et al. Figure 5 shows the LC spots (and a small amount of other proteins) arranged diagonally in the two-dimensional IEF gel. No additional spots have been generated below any original spot, suggesting that no chemical modification occurred during IEF.

The second method of examining the authenticity of the LC forms was that of Haeberle et al. (69), who claimed that inclusion of DTT

Figure 5. Two-dimensional IEF of myosin light chains prepared from crude aorta actomyosin phosphorylated for 5 min. Spots 0 through 4 of the LC are indicated.


1st dimension
4.5 ← - - - - - - - pH - - - - - - - → 5.4
2nd dimension
4.5 ← - - - - pH - - - - → 5.4
1
2
3
4

in the solution used to dissolve the muscle proteins for electrophoresis, and pre-electrophoresis of the anionic reducing agent thioglycolate into the gels eliminated charge modification of uterine LC and reduced the observed four spots to two (spots 3 and 4). Since DTT was already included in our IEF sample solution (p. 28), the only modification needed was the addition of thioglycolate to the cathode solution during pre-electrophoresis of the IEF gels. Gels were therefore pre-electrophoresed with and without 1 mM sodium thioglycolate in the cathode solution (20 mM NaOH) at 400 V for 1 h. Myosin light chains were electrophoresed in these gels and then in SDS gels as usual. After staining, the LC pattern of gels treated with thioglycolate was compared to that of regular gels. I found not only that the number of LC spots did not change by the thioglycolate treatment, but that the distribution of staining intensity among the spots did not change either. This is shown in Table I, which presents the distribution in two samples with different phosphorylation times and phosphorylation levels. In each sample the distribution is practically the same with or without thioglycolate. The fact that thioglycolate failed to eliminate or even change the amount of any LC spots adds to the evidence that these spots are not experimental artifacts.

B. Evidence for isoforms of the 20-kDa myosin light chain

Having shown that the multiple spots observed at the 20-kDa level are naturally occurring molecules, I examined their structural relationship. Although four spots are obtained with any of the five

TABLE I

EFFECT OF SODIUM THIOGLYCOLATE ON THE DISTRIBUTION OF THE MYOSIN LIGHT CHAIN FORMS OF PORCINE AORTA

Phosphorylation time (min)	Thioglycolate	Percent distribution[a]				
		spot 0	spot 1	spot 2	spot 3	spot 4
0.5	+	0	4 ± 0	15 ± 1	55 ± 2	26 ± 2
0.5	-	0	6 ± 2	14 ± 3	54 ± 2	26 ± 3
15	+	3 ± 1	10 ± 2	35 ± 1	37 ± 1	15 ± 1
15	-	3 ± 0	10 ± 2	36 ± 1	37 ± 2	14 ± 2

[a]Values are expressed as mean ± standard deviation of 3 determinations.

treatments described in section III.A, the number of spots can be smaller under conditions that favor dephosphorylation of the LC. Such conditions are the incubation in a nutrient-free medium, which depletes the muscle's ATP, and the incubation with EGTA, which chelates the existing Ca^{2+} and inactivates MLCK. When carotid arteries were homogenized in buffered 150 mM NaCl and were incubated at room temperature for 2 h, spot 1 disappeared (Table II). When the incubation was repeated in the presence of 1 mM EGTA, spot 3 also disappeared and only spots 2 and 4 were left at a proportion of 15 and 85%, respectively (Figure 6). The same result was obtained when intact carotid arteries were stored in glucose-free PSS in the coldroom for 3 days. It is therefore concluded that two of the four 20-kDa polypeptides are unphosphorylated.

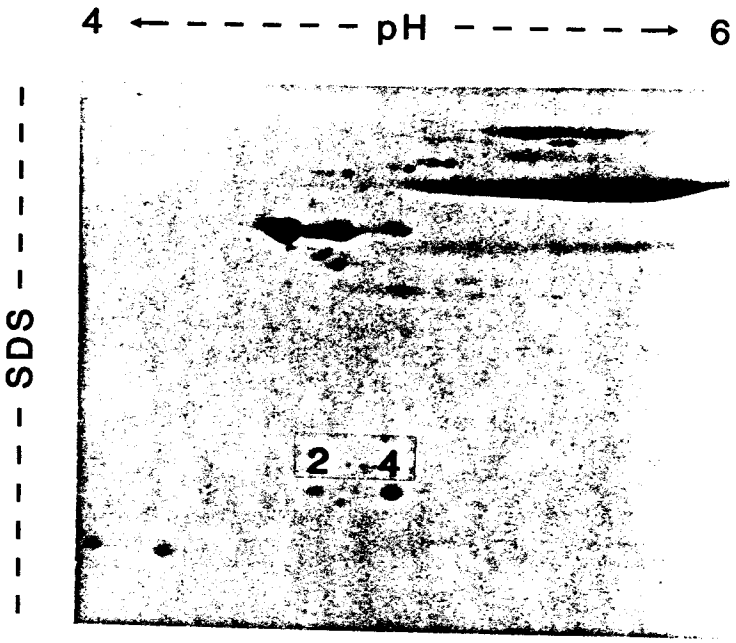
The two unphosphorylated polypeptides may be closely related variants of the LC or may be unrelated molecules that just happen to have very similar molecular masses and pIs. To distinguish between the two possibilities, I purified the two polypeptides and compared their structure by tryptic peptide mapping. Figure 7 outlines the main steps of the purification procedure described in detail in sections III.E, F.1, and G, as well as the protein yields. Briefly, the procedure consists of (a) homogenization of aortas in low ionic strength buffer to remove soluble proteins; (b) extraction of actomyosin with 0.6 M KCl, 10 mM ATP; (c) denaturation of actomyosin with 5 M guanidine.HCl and isolation of myosin light chains by 67% ethanol fractionation; (d) preparative SDS-PAGE to isolate the

TABLE II

DEPHOSPHORYLATION OF MYOSIN LIGHT CHAIN OF PORCINE CAROTID ARTERIES

Treatment	Percent distribution			
	spot 1	spot 2	spot 3	spot 4
Tissue homogenate in 150 mM NaCl, 25°C, 2 h	0	16	5	79
Tissue homogenate in 150 mM NaCl, 1 mM EGTA, 25°C, 2 h	0	15	0	85
Intact tissue in glucose-free PSS, 4°C, 3 days	0	15	0	85

Figure 6. Two-dimensional gel electrophoresis of proteins from homogenized carotid arteries, incubated with 1 mM EGTA for 2 h. Top: Coomassie blue-stained gel. Spots 2 and 4 of the LC are indicated. Bottom: densitometric tracing of the LC spots.


4 ← pH → 6
SDS
2 4

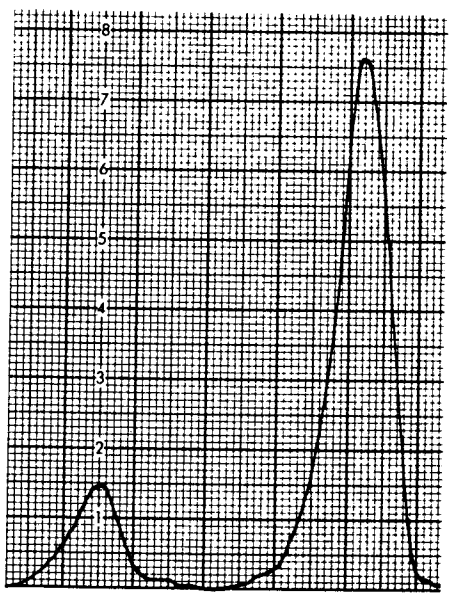
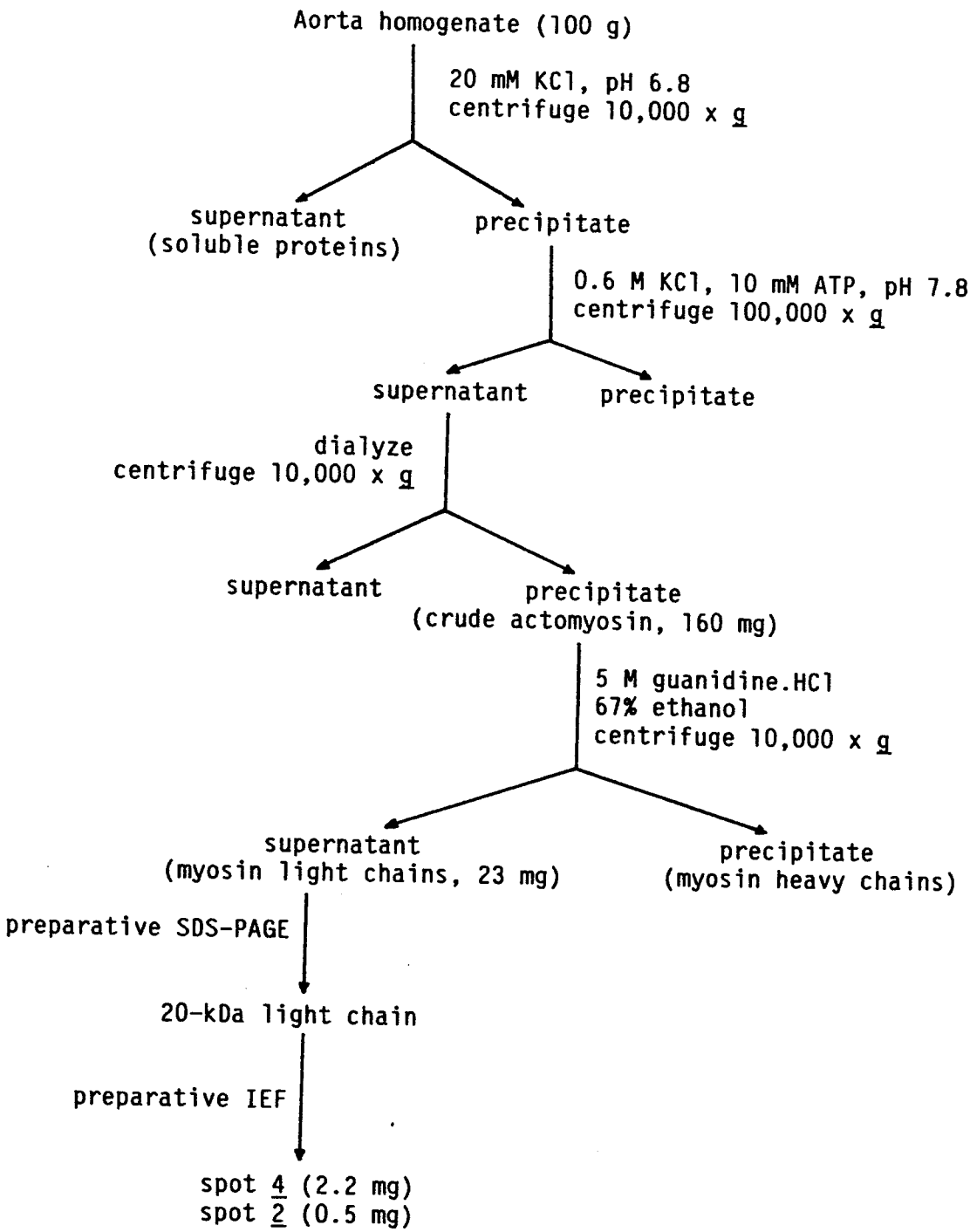

8
7
6
5
4
3
2
1

Figure 7. Purification scheme of the unphosphorylated LC forms of porcine aorta.


Aorta homogenate (100 g)
20 mM KCl, pH 6.8
centrifuge 10,000 x g
supernatant
(soluble proteins)
precipitate
0.6 M KCl, 10 mM ATP, pH 7.8
centrifuge 100,000 x g
supernatant
precipitate
dialyze
centrifuge 10,000 x g
supernatant
precipitate
(crude actomyosin, 160 mg)
5 M guanidine.HCl
67% ethanol
centrifuge 10,000 x g
supernatant
(myosin light chains, 23 mg)
precipitate
(myosin heavy chains)
preparative SDS-PAGE
20-kDa light chain
preparative IEF
spot 4 (2.2 mg)
spot 2 (0.5 mg)

LC; (e) preparative IEF of the LC to separate spots 2 and 4; and (f) extraction of the two forms from the polyacrylamide gel. The final yield was 0.5 and 2.2 mg per 100 g of wet tissue, respectively.

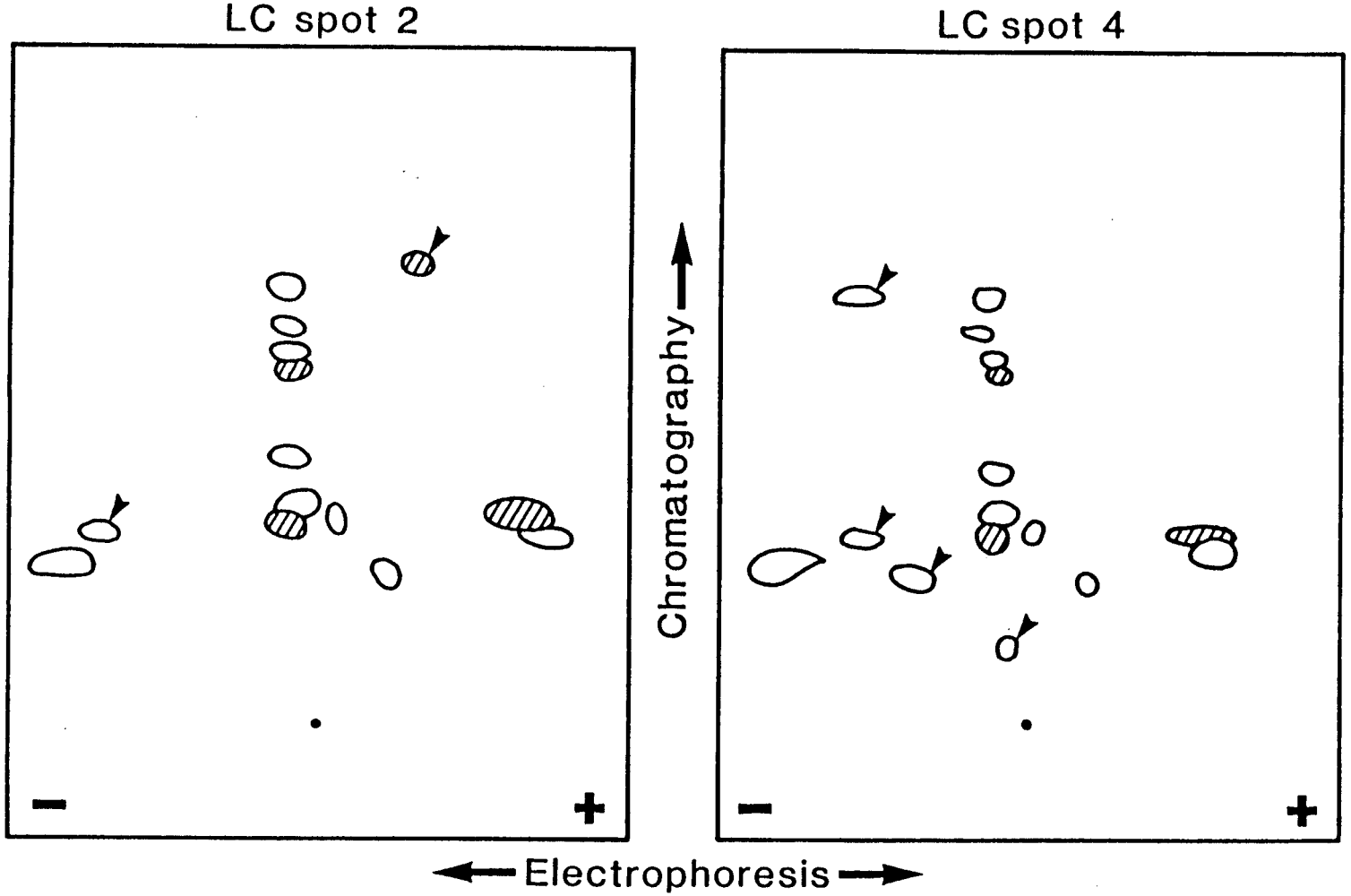
The tryptic peptide maps of spots 2 and 4 are presented in Figure 8. Spot 2 has fourteen tryptic peptides and spot 4 has sixteen tryptic peptides. Twelve of the peptides are at identical positions in the two maps, whereas two peptides of spot 2 are not present in the map of spot 4 and four peptides of spot 4 are not present in the map of spot 2. Three of the twelve common peptides are stained yellow by ninhidrin due to their proline content. Also one of the six peptides that are not shared by the two forms is yellow (in the map of spot 2). Most of the peptides did not migrate more than 2 cm from the point of application during electrophoresis, but they spanned two thirds of the distance covered by the chromatography solvent.

The great similarity of the two peptide maps in association with their clear differences suggests that spots 2 and 4 have similar but not identical structure, therefore they are isoforms of the LC.

C. Isoelectric points of the light chain forms

Isoelectric points were determined by coelectrophoresis of the LC forms with pI markers in IEF gels. This determination is not possible with total muscle proteins, because they contain much more tropomyosin than LC, and the LC spots are not discernible (see also p. 45). Therefore I used again the phosphorylated myosin light

Figure 8. Tryptic peptide maps of the unphosphorylated LC forms of porcine aorta. The patterns presented are tracings of ninhydrin-positive spots. Open spots are purple and cross-hatched spots are yellow. Arrowheads indicate differences between the two peptide maps. The points of sample application are indicated by full circles.


LC spot 2
LC spot 4
Chromatography
Electrophoresis
-
+
-
+

chains of aorta, which contain five LC spots and only traces of tropomyosin. One such determination is shown in Figure 9. Since all LC spots were between or very close to two of the three pI markers, that is soybean trypsin inhibitor (pI 4.55) and β-lactoglobulin A (pI 5.13), only they were used for calibration, and the pH gradient between them was assumed to be linear. None of the two marker proteins focused as a single band, therefore the band with the most intense staining was taken as having the pI quoted by the manufacturer. The results of the pI determination are presented in Table III. The five spots spread over 0.62 pH units and are spaced fairly uniformly (0.14-0.17 pH units from each other).

The pIs of the LC forms of carotid arteries were determined indirectly by comparison to the LC forms of aorta. Three hundred μg of carotid artery proteins, mixed with 40 μg of myosin light chains from aorta, were analyzed by two-dimensional gel electrophoresis. After staining, only four spots existed at the 20-kDa level. This means that the aorta LC spots and the carotid artery LC spots comigrate, therefore they have the same pIs.

D. Phosphate content of the myosin light chain of intact carotid arteries

Porcine carotid arteries were labeled with $^{32}P_i$ and were frozen at five different functional states as described in section III.A. Arterial proteins were analyzed by two-dimensional gel

Figure 9. Isoelectric point determination of the LC forms (spots 0 through 4) of porcine aorta. Top: pI markers. Bottom: myosin light chains prepared from actomyosin phosphorylated for 5 min. Middle: mixture of myosin light chains and pI markers.

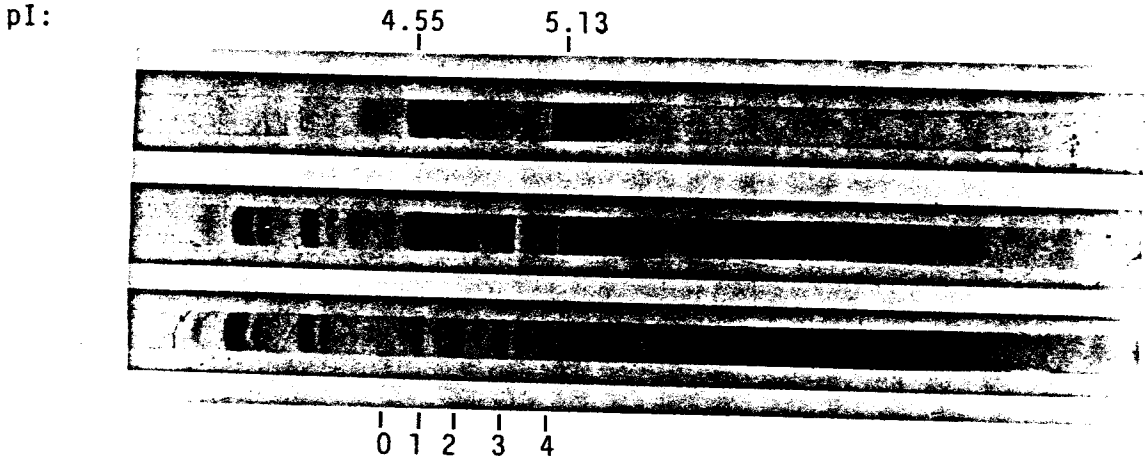

pI:
4.55
5.13
0 1 2 3 4

TABLE III

ISOELECTRIC POINTS OF THE MYOSIN LIGHT CHAIN FORMS OF PORCINE AORTA

Spot number	Isoelectric point[a]
0	4.44 ± 0.03
1	4.59 ± 0.01
2	4.73 ± 0.01
3	4.90 ± 0.03
4	5.06 ± 0.02

[a]Values are expressed as mean ± standard deviation of 4 determinations.

electrophoresis and the incorporation of [^{32}P]phosphate into each LC spot was determined as described in sections III.B and C, respectively. Data obtained from 66 gels have been compiled in Table IV. The results suggest that spot 4 contains no phosphate, whereas spots 3 and 1 contain approximately 1 mol of phosphate per mol of LC. This is in agreement with the observation that spots 1 and 3 disappear upon dephosphorylation of the LC (Table II) and suggests that they are the phosphorylated forms of spots 2 and 4, respectively.

The phosphate content of spot 2 is puzzling for the following reasons:

1. Based on the results of the dephosphorylation experiments, spot 2 is considered to be unphosphorylated LC; however, the data of Table IV and the autoradiograms of Figure 3 show that it contains considerable amount of [^{32}P]phosphate.

2. The mean phosphate content of spot 2 (0.65 mol/mol) is far from a stoichiometry of 1 mol/mol. Moreover, the experimental values showed great dispersion (which is evident from the high standard deviation relative to the mean value). Although the incorporation values for the other three spots showed no dependence on the total incorporation of [^{32}P]phosphate into the LC, the values for spot 2 depended on the total incorporation and generally increased with increasing total phosphorylation.

3. If spot 1 is produced from spot 2 by phosphorylation, and completely dephosphorylated LC contains 15% spot 2 (Table II), the

TABLE IV

INCORPORATION OF [^{32}P]PHOSPHATE INTO THE MYOSIN LIGHT CHAIN FORMS OF INTACT CAROTID ARTERIES

Spot number	mol [^{32}P]phosphate[a] / mol LC
1	1.19 ± 0.44
2	0.65 ± 0.47
3	0.83 ± 0.29
4	0.04 ± 0.04

[a]Values are expressed as mean ± standard deviation of data obtained from 66 gels.

sum of spots 1 and 2 should always be 15%. However, in most gels the sum of spots 1 and 2 was more than 15% (see Table V).

The above apparent peculiarities can be explained by accepting that muscles with medium or high level of phosphorylation, such as the muscles at resting tension or under stimulation, contain an additional phosphorylated form in spot 2. This phosphorylated form has the same pI as the unphosphorylated LC isoform, it is responsible for the radioactivity of spot 2, and its amount is related directly to the overall level of phosphorylation. To calculate the stoichiometry of the phosphorylated form in spot 2, one needs to know how much of spot 2 belongs to it. Since, as mentioned above, the sum of spots 1 and 2 was over 15% in most gels, the percentage in excess of 15, that is (spot 1 + spot 2 -15), should belong to the phosphorylated form in spot 2. This difference ranged from 0 to 8%. In the gels that it was 2% or more (40 of the 66), this percentage was used to calculate the pmol of LC in the phosphorylated form of spot 2 as described on p. 31. By dividing the pmol of [^{32}P]phosphate in spot 2 by the pmol of LC in the phosphorylated part only, a stoichiometry of 2.1 ± 0.6 mol [^{32}P]phosphate/mol LC (n=40) was obtained. This suggests that the phosphorylated form in spot 2 is actually diphosphorylated LC produced by further phosphorylation of spot 3. Therefore spot 2 is a mixture of unphosphorylated minor isoform and diphosphorylated major isoform. The two forms have the same pI because the difference of two charges between the two unphosphorylated isoforms is eliminated by the attachment of two phosphate groups to the major isoform.

Since the tryptic peptide maps of the two LC isoforms show that they have great structural similarity, it is reasonable to ask whether the minor isoform can be diphosphorylated as well. The answer is, indeed, the minor isoform can be diphosphorylated, but its amount is so low that it cannot be detected by the Coomassie blue stain. However, a radioactive spot more acidic than spot 1 was visible in overexposed autoradiograms of gels of highly phosphorylated arteries. Conclusive evidence for diphosphorylation of the minor LC isoform will be presented in section G.

Based on the identification of the four spots described above, the total phosphate content, P, of the LC can be calculated from the percent distribution of staining intensity among the spots as the sum of the monophosphorylated spots 1 and 3 plus two times the diphosphorylated constituent of spot 2. This is expressed by Equation 2:

$$P = \frac{\text{spot } 1 + 2 \times (\text{spot } 1 + \text{spot } 2 - 15) + \text{spot } 3}{100} \quad \frac{\text{mol phosphate}}{\text{mol LC}}$$

(Equation 2)

If Equation 2 is correct, the phosphate content calculated from the percent distribution of staining intensity should be the same as the phosphate content calculated from the sum of the radioactivity of the LC spots. This comparison is made in Table V for each of the five treatments that the arteries were subjected to (first column).

TABLE V

MYOSIN LIGHT CHAIN PHOSPHORYLATION IN ^{32}P-LABELED INTACT CAROTID ARTERIES

Treatment	Percent distribution[a]				mol phosphate / mol LC	mol [^{32}P]phosphate / mol LC	Number of samples
	spot 1	spot 2	spot 3	spot 4			
None	4 ± 1	13 ± 1	24 ± 4	59 ± 6	0.32 ± 0.07	0.28 ± 0.08	7
50 μM Norepinephrine	6 ± 1	13 ± 1	39 ± 3	42 ± 4	0.52 ± 0.05	0.49 ± 0.04	4
100 mM KCl	6 ± 2	11 ± 2	49 ± 7	34 ± 6	0.59 ± 0.06	0.64 ± 0.20	10
Stretch	5 ± 2	11 ± 1	48 ± 7	36 ± 8	0.56 ± 0.11	0.50 ± 0.22	7
Stretch and 100 mM KCl	7 ± 1	11 ± 3	58 ± 3	24 ± 5	0.71 ± 0.08	0.69 ± 0.12	10

[a]All values are expressed as mean ± standard deviation. Each sample was analyzed in 2-5 gels.

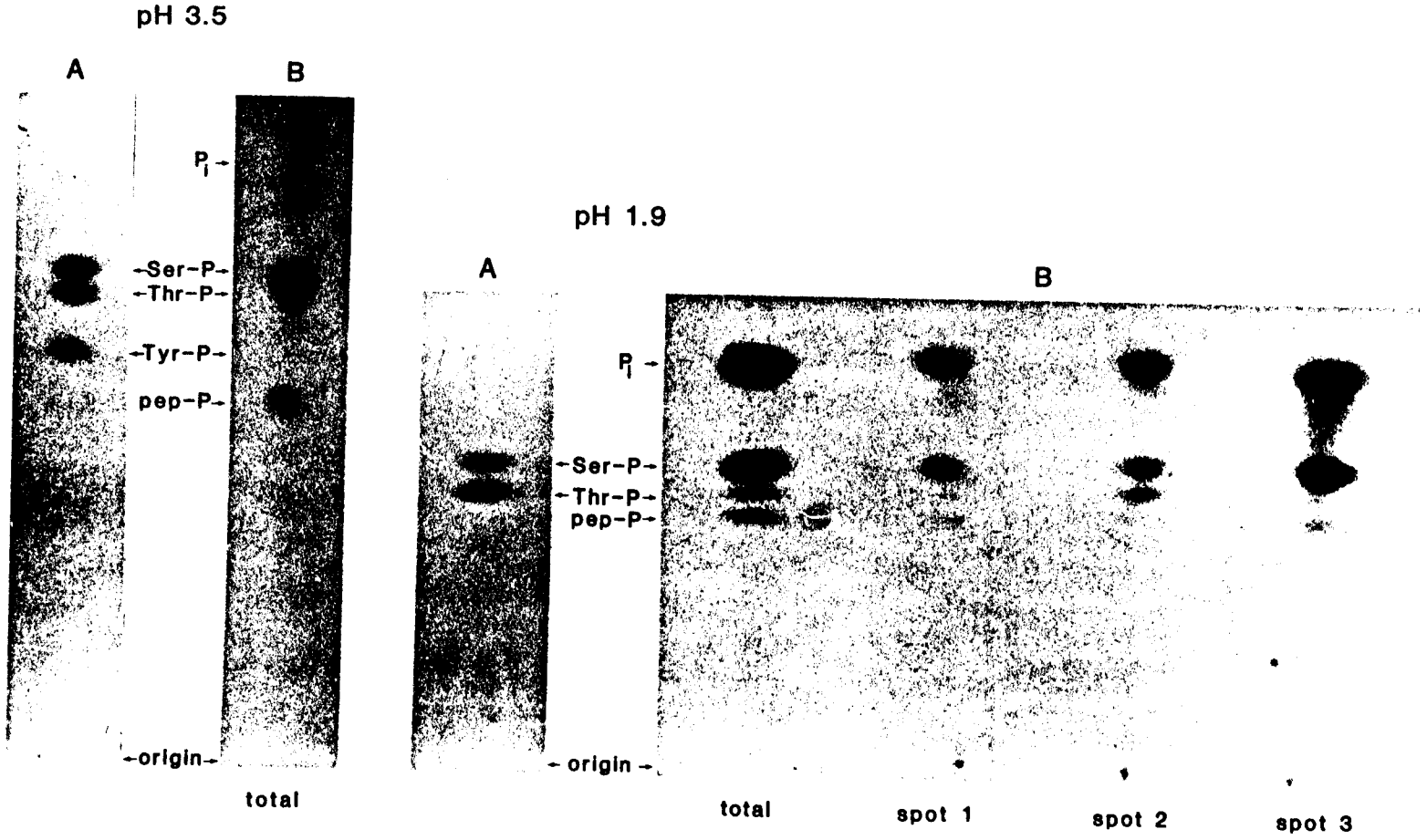
The second column presents the distribution of staining intensity among the four spots and shows an increase in spots 1 and 3 upon stimulation of the muscle. The data of this column were used in Equation 2 to produce the values of the third column. The fourth column has been constructed from the radioactivity data. The two sets of values in the third and fourth column are in agreement with each other within experimental error.

E. Phosphoamino acid analysis of myosin light chain in intact carotid arteries

Phosphoamino acid analysis of phosphoproteins consists of two parts: identification and quantitation. Both are performed by labeling the phosphoprotein(s) of interest with ^{32}P because of the higher sensitivity of the method compared to the analytical determination of phosphoamino acids. Radioactive phosphoamino acids can be detected in acid hydrolyzates of [^{32}P]phosphoproteins because the hydrolysis of phosphomonoester bonds is considerably slower in acid than the hydrolysis of peptide bonds (103). After hydrolysis of the protein, the amino acids are separated by a variety of electrophoretic or chromatographic procedures (102).

The identification of phosphoamino acids in the LC of ^{32}P-labeled carotid arteries is presented in Figure 10. The three phosphorylhydroxyamino acids, Ser-P, Thr-P, and Tyr-P, can be separated by electrophoresis at pH 3.5 (A). Thin-layer electrophoresis of acid hydrolyzates of ^{32}P-labeled LC extracted from SDS-polyacrylamide gels, and subsequent autoradiography of the

Figure 10. Identification of phosphoamino acids in the LC of a ^{32}P-labeled carotid artery, which was stimulated by stretch and addition of 100 mM KCl. A: ninhydrin stain patterns of marker phosphoamino acids. B: autoradiograms of acid hydrolyzates of total LC and individual radioactive spots (at pH 1.9). Pep-P stands for phosphopeptide.


pH 3.5
A
B
P_i
Ser-P
Thr-P
Tyr-P
pep-P
origin
total
pH 1.9
A
B
P_i
Ser-P
Thr-P
pep-P
origin
total
spot 1
spot 2
spot 3

cellulose sheet revealed radioactivity in Ser-P and Thr-P and no radioactivity in Tyr-P (B). Two more radioactive spots were visible. One corresponds to P_i, which is the product of hydrolysis of phosphomonoester bonds, and the other corresponds to a phosphopeptide, which is the product of incomplete hydrolysis of peptide bonds. Since Ser-P and Thr-P are not separated completely at pH 3.5, and since no Tyr-P is present in the LC, I chose to electrophorese the hydrolyzates at pH 1.9, at which Ser-P and Thr-P are clearly separated (Tyr-P comigrates with Thr-P at pH 1.9). Electrophoresis of total LC hydrolyzates confirmed the presence of Ser-P and Thr-P. When the individual radioactive spots were extracted from two-dimensional gels and were subjected to hydrolysis and electrophoresis, I found that the monophosphorylated spots 1 and 3 contain only Ser-P, whereas spot 2, which contains diphosphorylated LC, has both Ser-P and Thr-P at comparable amounts. These results were independent of muscle treatment.

Now the question arises: how much Ser-P and Thr-P is there in the LC and how does their amount change as the total phosphate content of the LC changes upon stimulation of the arteries? Quantitation of phosphoamino acids is complicated by the different rates of release from the protein and the different rates of degradation during acid hydrolysis (102,103). Because of this, the radioactivity of the phosphoamino acid spots does not reflect their exact amounts in the protein. However, in the case of the LC the quantitation of Ser-P and Thr-P becomes possible by the results of

the identification of the phosphoamino acids present in spots 1, 2, and 3 (Figure 10). According to these results, the entire radioactivity of spots 1 and 3 and one half the radioactivity of spot 2 belongs to Ser-P, whereas Thr-P accounts for one half the radioactivity of spot 2 only. Consequently, the data that were used to determine the phosphate content of each LC spot in the previous section can now be used to calculate the phosphoamino acid content of the LC as well. The results of these calculations are summarized in Table VI. Here the data used to construct Table IV have been grouped according to treatment of the arteries (first column). The second column was made by adding the radioactivity of the four LC spots. The third column was made according to the equation:

$$\frac{\text{mol } [^{32}P]\text{Ser-P}}{\text{mol } [^{32}P]\text{Thr-P}} = \frac{\text{cpm of (spot 1 + 1/2 x spot 2 + spot 3)}}{\text{1/2 x cpm of spot 2}}$$

(Equation 3)

The mol of each phosphoamino acid per mol of LC (fourth and fifth column) were calculated from the total phosphate content of the LC (second column) and from the molar ratio of Ser-P to Thr-P (third column).

The amount of Ser-P is much higher than the amount of Thr-P in all cases. Their molar ratio is between 11 and 12 for arteries frozen at resting tension or after stimulation by norepinephrine or KCl. Differences among the three treatments are not significant ($P > 0.3$, Student's t test). The same ratio decreases to approximately

TABLE VI

PHOSPHOAMINO ACID CONTENT OF MYOSIN LIGHT CHAIN IN INTACT CAROTID ARTERIES

Treatment	mol $[^{32}]$phosphate[a] / mol LC	mol $[^{32}P]$Ser-P / mol $[^{32}P]$Thr-P	mol $[^{32}P]$Ser-P / mol LC	mol $[^{32}P]$Thr-P / mol LC	Number of gels
None	0.27 ± 0.09	11.2 ± 2.2	0.25 ± 0.08	0.02 ± 0.01	19
50 μM Norepinephrine	0.49 ± 0.06	12.1 ± 2.6	0.45 ± 0.05	0.04 ± 0.01	10
100 mM KCl	0.68 ± 0.21	11.3 ± 1.7	0.62 ± 0.19	0.06 ± 0.02	16
Stretch	0.55 ± 0.21	8.4 ± 0.5	0.49 ± 0.19	0.06 ± 0.02	8
Stretch and 100 mM KCl	0.71 ± 0.08	8.0 ± 0.8	0.63 ± 0.07	0.08 ± 0.01	4
Unstretched arteries		11.4 ± 2.1			45
Stretched arteries		8.3 ± 0.6			12

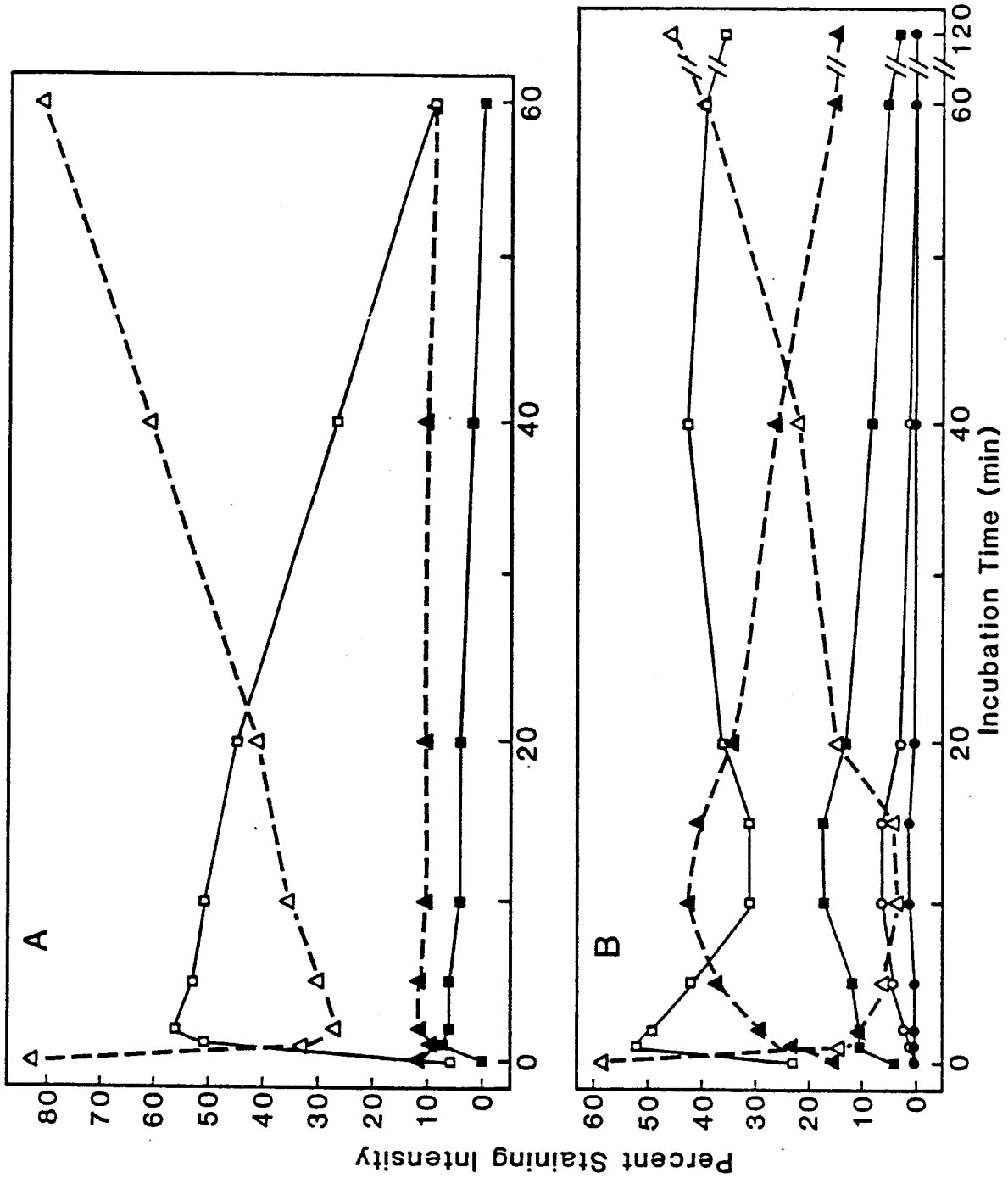
[a]All values are expressed as mean ± standard deviation.

8 after stretching the arteries, with no significant difference between stretching alone and stretching combined with KCl stimulation ($P > 0.4$). However, the difference between any of the three treatments of the first group and any of the two treatments of the second group, or between unstretched arteries collectively and stretched arteries collectively (lower part of Table VI) is significant ($P < 0.001$). These data show that chemical treatment of the arteries causes proportional changes in the two phosphoamino acids and does not change their ratio significantly, whereas application of mechanical stress increases the amount of Thr-P more than it increases the amount of Ser-P.

F. Phosphorylation of myosin by endogenous kinases in vitro

The appearance of a fifth LC spot upon phosphorylation of crude aorta actomyosin (p. 45) prompted me to investigate the LC phosphorylation pattern in vitro. Two different systems, aorta homogenate and crude actomyosin, were incubated under the same conditions: at room temperature in a low ionic strength buffer that maintains myosin in its native filamentous form. Phosphorylation was initiated by addition of 0.1 mM Ca^{2+} to activate MLCK, 1 mM ATP, and 5 mM Mg^{2+}, since MgATP is the true substrate in all biochemical reactions involving ATP hydrolysis. Phosphorylation was terminated at various times with 5% TCA and the proteins of each preparation were separated by two-dimensional gel electrophoresis. The percent distribution of staining intensity among the LC spots was determined by scanning densitometry and was plotted as a function of incubation time. Results are shown in Figure 11.

Figure 11. Time course of LC phosphorylation in porcine aorta homogenate (A) and crude actomyosin (B) by endogenous kinases. (•), spot -1; (○), spot 0; (■), spot 1; (▲), spot 2; (□), spot 3; (△), spot 4.


A
B
Percent Staining Intensity
Incubation Time (min)
0
10
20
30
40
50
60
70
80
0
20
40
60
120

In the tissue homogenate (panel A) and before addition of Ca^{2+}, Mg^{2+}, and ATP the LC consists mainly of the unphosphorylated spots 4 and 2 (0 min). Upon addition of these reagents a rapid decrease in the amount of spot 4 and a rapid increase in the amount of the phosphorylated spots 3 and 1 is observed. Phosphorylation is maximal within 2 min of incubation. The distribution of staining intensity at 2 min (6,11,56,and 27% from spot 1 to spot 4) is very similar to the highest phosphorylation levels obtained with intact carotid arteries (Table V). This suggests an analogy between stimulation of the intact smooth muscle and addition of ATP to tissue homogenate. As the incubation proceeds, the changes in LC distribution are reversed, that is, spot 4 increases and spots 3 and 1 decrease. Dephosphorylation of the LC is much slower than the phosphorylation and can be explained by the depletion of ATP and the action of phosphatases. After 60 min of incubation the distribution of LC forms has returned to that at 0 min.

The staining intensity of spot 2 remained low and relatively constant throughout the incubation of the aorta homogenate. This reflects the dual nature of spot 2 that was described in section D. During phosphorylation of the LC the minor isoform (spot 2) is converted to spot 1, whereas the major isoform is diphosphorylated and compensates for the loss in spot 2. During dephosphorylation the opposite changes happen.

In the crude actomyosin preparation (panel B) the LC has a moderate degree of phosphorylation even before addition of

Ca^{2+}, Mg^{2+}, and ATP. The distribution at 0 min (4, 15, 23, and 58%) resembles the distribution in carotid arteries frozen at resting tension (Table V). The higher starting level of phosphorylation (as compared to the tissue homogenate) can be explained by the presence of 10 mM ATP in the myosin extraction buffer (p. 33). This served as substrate for MLCK, but the absence of added Ca^{2+} and Mg^{2+}, the presence of 2 mM EGTA and 1 mM EDTA in the extraction buffer, and the low temperature of the preparation kept the phosphorylation level of LC low. Upon addition of Ca^{2+}, Mg^{2+}, and ATP there is a rapid decline of spot 4 and a rapid rise of spots 3 and 1, much in the same way as in the aorta homogenate (panel A). Spot 3 reaches a peak of 52% at 1 min of incubation, which resembles the peak in the homogenate. The similarities end here, however, because spot 2 rises to such an extent in the actomyosin (maximum 42%), that it becomes the major LC spot between approximately 6 and 19 min of incubation. As I showed in section D, spot 2 is a mixture of unphosphorylated minor isoform and diphosphorylated major isoform. Since the minor isoform is only 15% of the LC, the high amount of spot 2 in actomyosin must be due to a high degree of diphosphorylation, which did not exist in the intact tissue or in the homogenate. This is also suggested by the interconversion of spots 3 and 2 seen in the time course of phosphorylation: spot 3 decreases between 1 and 10 min as spot 2 increases, and then it increases slowly between 15 and 40 min as spot 2 decreases.

Other striking events also take place during incubation of the actomyosin. Spot 1 reaches 17% of the total LC at 10 and 15 min, which is much higher than the maximum 7% obtained with intact arteries (Table V) or homogenate (Figure 11, panel A), and which alone is more than the amount of the minor LC isoform. This suggests that not only spot 2, but spot 1 as well, may contain part of the major isoform (in what phosphorylation state?). Then the fifth LC spot that was first mentioned on p. 45 appears between 1 and 40 min of incubation. Spot 0 reaches 6% of the LC. Finally, a sixth spot completes the LC pattern at 10 and 15 min. This spot, named spot -1, comprises an average 1% of the LC. At the peak of phosphorylation (between 10 and 15 min) spot 4 almost disappears.

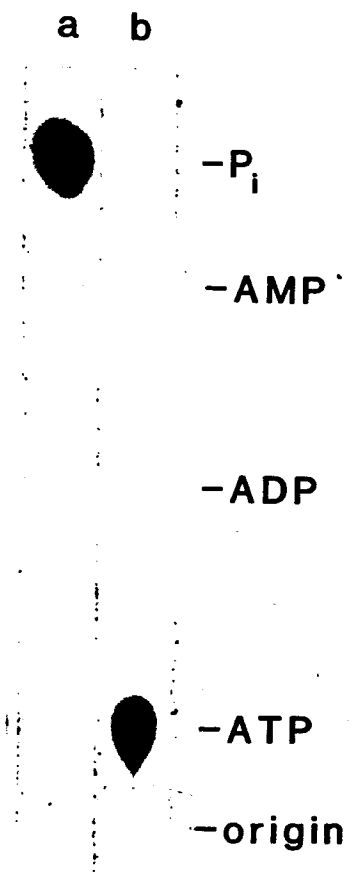
Dephosphorylation follows the peak of LC phosphorylation. Spots -1 and 0 disappear, spots 1, 2, and 3 decrease, and spot 4 increases. Dephosphorylation is slower in the actomyosin than in the tissue homogenate. Even after 2 h of incubation a considerable degree of phosphorylation persists (distribution 3, 15, 36, and 46% from spot 1 to spot 4). This may be due to loss of LC phosphatase activity during the preparation of actomyosin.

The data presented in this section show major differences in the LC phosphorylation pattern between aorta homogenate and crude actomyosin upon incubation with ATP. These differences are due to the activation of an extraordinary LC kinase activity during the isolation of crude actomyosin.

G. Phosphate content of the myosin light chain forms of aorta actomyosin

In section D the stoichiometry of the phosphate content of the LC spots of intact carotid arteries was determined. Because spot 2 consisted mostly of unphosphorylated LC, a stoichiometry of 2 mol phosphate/mol LC for the phosphorylated form in that spot was deduced only indirectly. As shown in the previous section, spot 2 rises to almost three times the total amount of the minor isoform when actomyosin is incubated with ATP, and this should permit direct calculation of the stoichiometry of the phosphorylated form in spot 2. For this purpose and in order to determine the nature of the new spots -1 and 0, I decided to label the LC of crude aorta actomyosin with ^{32}P. Contrary to the intact tissue, which can synthesize [γ-^{32}P]ATP from ADP and [^{32}P]P_i, the actomyosin preparation is incapable of such synthesis, therefore [γ-^{32}P]ATP has to be supplied. Inexpensive γ-^{32}P-ATP was prepared enzymatically through the first substrate level phosphorylation of glycolysis. The yield of the reaction was examined by thin-layer anion exchange chromatography on polyethyleneimine-cellulose using 1.0 M LiCl for development (Figure 12). The system separates ATP, ADP, AMP, and P_i. Autoradiography of the developed sheet showed that most of the $^{32}P_i$ added to the reaction mixture had been converted to γ-^{32}P-ATP. No radioactivity was detected in any other nucleotide spot. By liquid scintillation counting it was determined that 97.7% of the total radioactivity had been incorporated into ATP.

Figure 12. Thin-layer anion exchange chromatography of $^{32}P_i$ (a) and reaction mixture after incubation of $^{32}P_i$ with GammaPrep-A (b).


a
b
-P_i
-AMP
-ADP
-ATP
-origin

This value was used for accurate determination of the specific radioactivity of [γ-^{32}P]ATP, which was prepared by isotopic dilution of the reaction mixture with a stock ATP solution, the concentration of which had been determined spectrophotometrically.

The [γ-^{32}P]ATP thus prepared was used as substrate for the phosphorylation of crude actomyosin by endogenous kinases. Figure 13 presents a two-dimensional gel of an actomyosin sample phosphorylated for 15 min. Six LC spots are visible in the Coomassie blue-stained gel (top) and in the densitometric tracing (bottom). Five of the six spots are radioactive; spot 4 is the only non-radioactive spot (middle).

The mol of [^{32}P]phosphate incorporated per mol of each LC spot were calculated as in the case of carotid arteries, with two differences. First, the specific radioactivity of [γ-^{32}P]ATP (instead of [^{32}P]phosphocreatine) was used to calculate the pmol of [^{32}P]phosphate in each LC spot. Second, whereas the myosin content of carotid artery proteins was available in the literature, the myosin content of my actomyosin preparation had to be determined. This was done for each sample by scanning densitometry of 7.5% polyacrylamide gels, which allow migration of all the proteins of the actomyosin preparation into the gel, as opposed to 15% polyacrylamide gels, which exclude MHC. As shown in Figure 14, actin and MHC are the major polypeptides of crude actomyosin, usually comprising 41 and 17% of the proteins, respectively. A substantial amount of polypeptides of molecular mass less than 40

Figure 13. Two-dimensional polyacrylamide gel electrophoresis of crude aorta actomyosin incubated with 1 mM [γ-^{32}P]ATP for 15 min. Top: Coomassie blue-stained gel. Spots -1 through 4 of the LC are indicated. Middle: autoradiogram of the gel. Bottom: densitometric tracing of the LC spots in the Coomassie blue-stained gel.

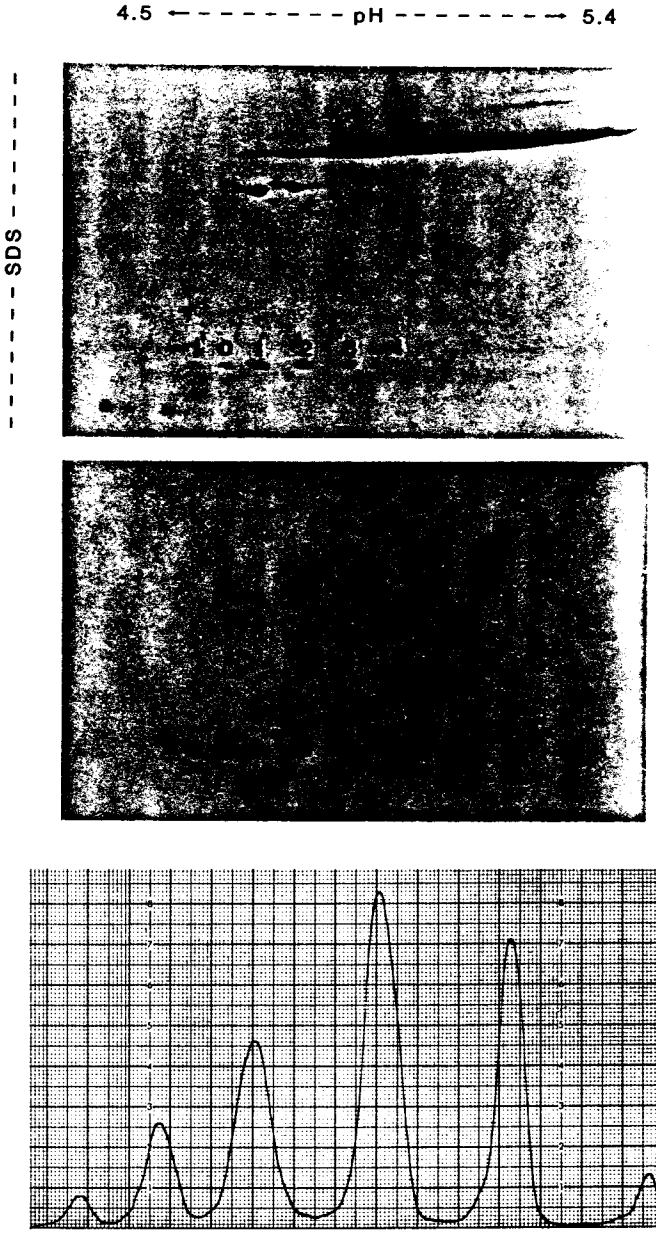
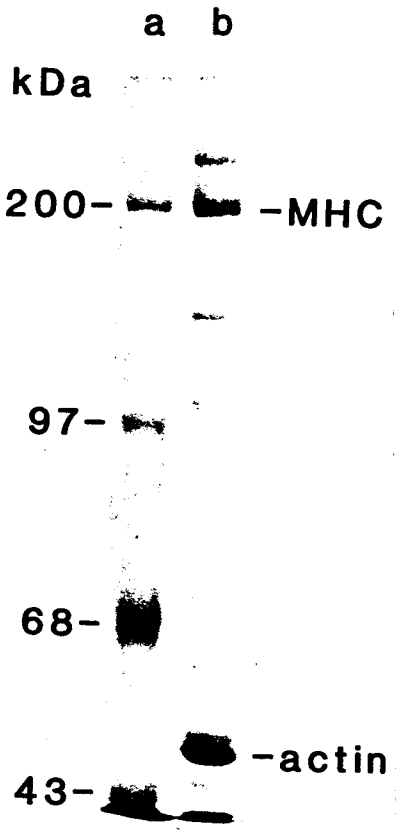

4.5 ← pH → 5.4
SDS

Figure 14. Coomassie blue stain of SDS-polyacrylamide gel used for determination of the myosin content of crude aorta actomyosin (7.5% polyacrylamide). Left lane: molecular mass standards. Right lane: Actomyosin sample.


a
b
kDa
200-
-MHC
97-
68-
-actin
43-

kDa are not resolved in the 7.5% polyacrylamide gel and migrate with the bromphenol blue tracking dye. Tropomyosin and myosin light chains are included in these polypeptides. From the MHC content, the LC content of each actomyosin sample was determined as described on p. 41.

Table VII presents the mol of [^{32}P]phosphate incorporated per mol of each LC spot. Comparison of its data to those from intact carotid arteries (Table IV) shows similarity only in spots 4 and 3, which correspond to unphosphorylated and monophosphorylated LC, respectively. The stoichiometry of spot 2 is approximately 2 mol of [^{32}P]phosphate per mol of LC. This confirms the evidence for the existence of diphosphorylated LC presented on p. 65. In intact carotid arteries spot 2 contains more unphosphorylated minor isoform than diphosphorylated major isoform, therefore its phosphate content is closer to 0 than to 2 mol/mol. On the contrary, in aorta actomyosin diphosphorylated LC constitutes the overwhelming majority of spot 2, bringing its phosphate content very close to 2 mol/mol.

The phosphate content of spot 1 is surprising, since it points to a stoichiomentry of 3 mol phosphate/mol LC. Spot 1 was considered monophosphorylated minor isoform in intact carotid arteries, however in actomyosin it is evident that spot 1 consists mainly of triphosphorylated LC. This shows that the diphosphorylated major isoform in spot 2 can be further phosphorylated to become part of spot 1.

TABLE VII

INCORPORATION OF [^{32}P]PHOSPHATE INTO THE MYOSIN LIGHT CHAIN FORMS OF AORTA ACTOMYOSIN

Spot number	mol [^{32}P]phosphate[a] / mol LC
0	2.27 ± 0.43
1	2.70 ± 0.24
2	1.70 ± 0.17
3	0.92 ± 0.10
4	0.10 ± 0.17

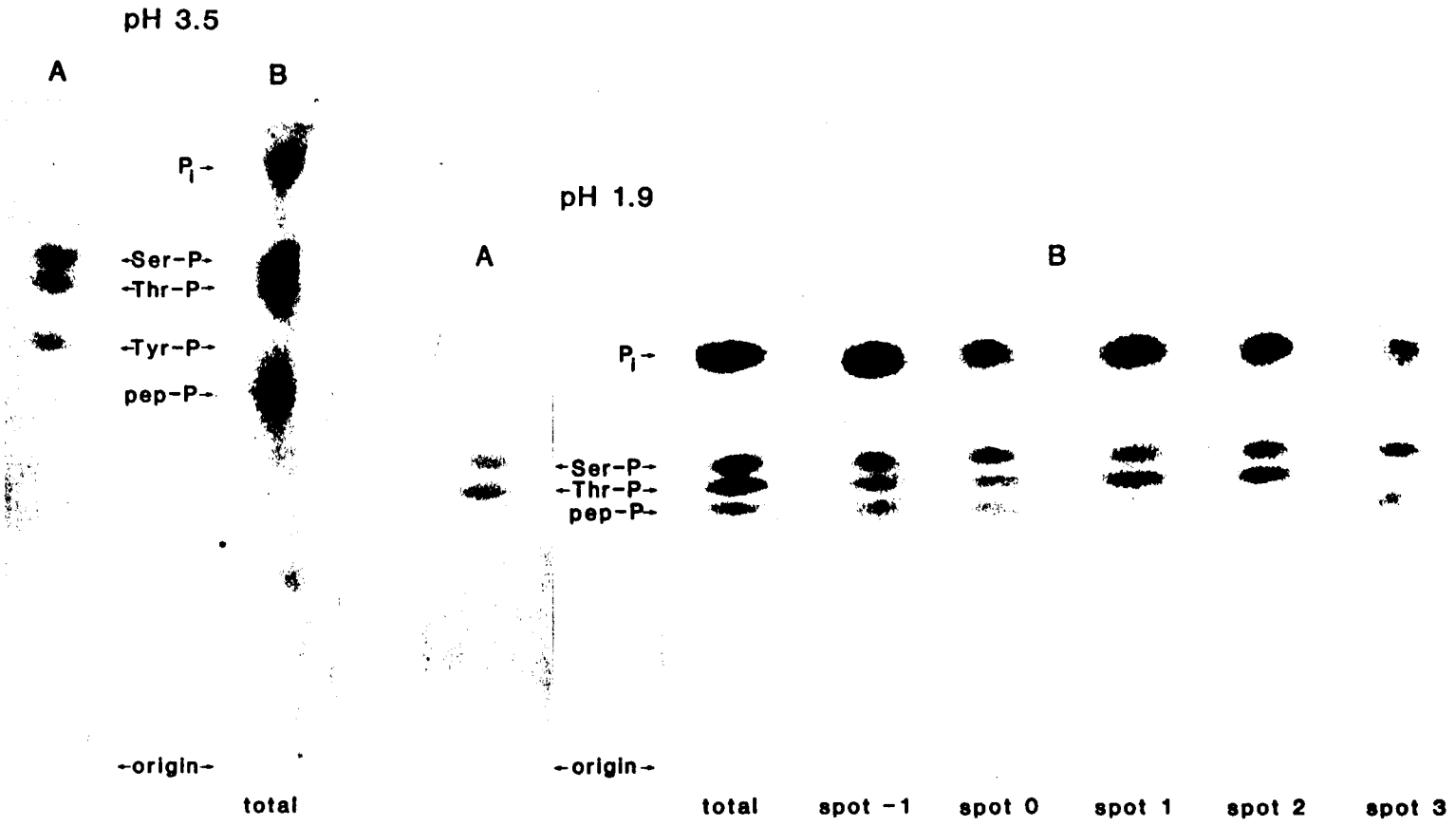
[a]Values are expressed as mean ± standard deviation of data obtained from 14 gels.

The phosphate content of spot 0 is approximately 2 mol/mol, thus confirming the hypothesis that, like the major isoform, the minor isoform can be diphosphorylated as well (p.66). Finally, the stoichiometry of [^{32}P]phosphate incorporation into spot -1 could not be determined, because its low staining intensity (1 ± 1% of the total LC) introduced a high degree of uncertainty into the calculations. Despite its low staining intensity, however, spot -1 contained considerable amount of radioactivity. Based on the fact that both LC isoforms can be diphosphorylated and the major isoform can be triphosphorylated, it is reasonable to assume that spot -1 represents the triphosphorylated minor isoform.

H. Phosphoamino acid analysis of myosin light chain in aorta actomyosin

The identification of the phosphoamino acids present in the total LC and in each of the five radioactive LC spots of aorta actomyosin incubated with [γ-^{32}P]ATP was performed similarly to the carotid arteries. Results are shown in Figure 15. Electrophoresis at pH 3.5 of acid hydrolyzates of LC extracted from SDS-polyacrylamide gels showed the presence of Ser-P, Thr-P, and no Tyr-P, in agreement with the results from carotid arteries (Figure 10). Subsequent analysis of the individual spots was performed at pH 1.9, at which Ser-P and Thr-P are clearly separated. The monophosphorylated spot 3 contains Ser-P and a small amount of Thr-P. This shows that it is possible for threonine to be phosphorylated first in the LC in the presence of high LC kinase

Figure 15. Identification of phosphoamino acids in the LC of aorta actomyosin phosphorylated for 15 min with 1 mM [γ-^{32}P]ATP. A: ninhydrin stain patterns of marker phosphoamino acids. B: autoradiograms of acid hydrolyzates of total LC and individual radioactive spots (at pH 1.9). Pep-P stands for phosphopeptide.


pH 3.5
A
B
Pi
Ser-P
Thr-P
Tyr-P
pep-P
origin
total
pH 1.9
A
B
Pi
Ser-P
Thr-P
pep-P
origin
total
spot -1
spot 0
spot 1
spot 2
spot 3

activity, but to a lower extent than serine. The diphosphorylated spot 2 contains both Ser-P and Thr-P at comparable amounts, in agreement with the carotid arteries. Spot 1 also contains Ser-P and Thr-P. The discrepancy with the intact arteries (where spot 1 contains only Ser-P) is due to the fact that in actomyosin spot 1 consists mainly of triphosphorylated LC, which will certainly contain the two phosphoamino acids of diphosphorylated LC. Spots 0 and -1 also contain both Ser-P and Thr-P.

What amino acid in the LC is the acceptor of the third phosphate group? This can be determined from the radioactivity of each phosphoamino acid in the triphosphorylated spots 1 and -1. It was mentioned (p. 71) that the radioactivity of the phosphoamino acid spots is not an accurate measure of their actual amounts in the protein because of their different rates of release and degradation. However, these differences are not so great as to conceal simple stoichiometries like 1:1 in a diphosphorylated protein or 2:1 in a triphosphorylated protein (it can be calculated, for example, from the data of reference 103 that, if one starts with equal amounts of Ser-P and Thr-P, after 2 h of acid hydrolysis their ratio will have changed merely to 0.95). I determined first the molar ratio of Ser-P to Thr-P in the diphosphorylated spots 2 and 0 by excising the two phosphoamino acid spots (with the flexible plastic backing) from four cellulose sheets, adding scintillation fluid, and measuring their radioactivity. Their ratio was found to be 1.0 ± 0.2 ($n = 8$), showing that one mol of diphosphorylated LC

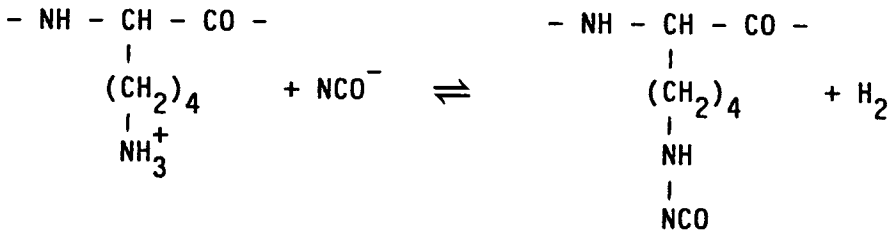
contains one mol of Ser-P and one mol of Thr-P. Then I determined the ratio of Ser-P to Thr-P in spots 1 and -1 and obtained a value of 1.1 ± 0.4 (n = 8). This is far from either 2, which would suggest that the third phosphate is esterified to serine, or 0.5, which would identify threonine as the acceptor of the third phosphate. The ratio of 1.1 may be due to experimental error, or it may indicate that a serine and a threonine residue in the LC are almost equally available for attachment of the third phosphate group.

V. DISCUSSION

Multiple forms of the 20-kDa myosin light chain of smooth muscle were first reported five years ago (64), and have since been detected in other laboratories (69,78,88). Several possibilities were raised to explain this multiplicity. The simplest explanation is that additional forms are produced from phosphorylated and unphosphorylated LC by chemical modification during IEF. It is known (93) that isocyanate formed by decomposition of urea according to the reaction

$$(H_2N)_2CO \rightleftharpoons NH_4^+ + NCO^-$$

may cause carbamylation of proteins:



lysine residue

This eliminates one positive charge per isocyanate ion at physiological pH and decreases the pI of the protein. Another possible modification is the oxidation of cysteine to cysteic acid (93) which can be caused by atmospheric oxygen or by the persulfate used for gel polymerization (69). This also would shift the pI to a lower value.

The following arguments speak against the possibility of artifactual charge modification in the LC:

1. All the precautions suggested by O'Farrell (93) to prevent carbamylation are taken routinely in this laboratory: All urea solutions are stored as frozen aliquots; ampholytes are present in all urea solutions which contact the protein (ampholytes contain reactive amines); once the protein sample is dissolved in a urea solution, it is loaded on the IEF gel as soon as possible; and the IEF gels are pre-electrophoresed to remove isocyanate and persulfate.

2. The gel electrophoretic procedure employed in this work has also been used in this laboratory to analyze skeletal muscle proteins (104) and uterine smooth muscle proteins (70). Although the LC of intact smooth muscle (either arterial or uterine) is resolved in four spots, the phosphorylatable myosin light chain of skeletal muscle is resolved in two spots only (one phosphorylated and one unphosphorylated). Such a systematic difference between skeletal and smooth muscle cannot be attributed to experimental artifact within the same laboratory, but it must be characteristic of myosin itself.

3. Additional precautions suggested by Haeberle et al. (69) to prevent oxidation are the use of DTT with the proteins and pre-electrophoresis of thioglycolate into the gels to reduce any persulfate that has not been used in the polymerization of acrylamide and is not removed by regular pre-electrophoresis. Dithiothreitol is always present in our solutions. Consequently, I

tested whether pre-electrophoresis of IEF gels in the presence of thioglycolate would have any effect on the LC pattern of arterial smooth muscle. Contrary to the results of Haeberle et al., the LC pattern did not change as a result of the thioglycolate treatment (Table I).

4. Driska et al. (64) based their assessment of the additional LC spots as artifacts on the finding that refocusing the protein contained in the major unphosphorylated or phosphorylated LC spot gave "an IEF gel pattern exhibiting the family of more acidic variants, although in small amounts". This result, however, may be due to chemical modification of the LC after the electrophoresis and during Coomassie blue staining and extraction of the protein from the gel. The best way to test for chemical modification during IEF is the two-dimensional IEF system of Gagelmann et al. (88), which refocuses each LC spot directly from the IEF gel without any treatment that might introduce artifact. Both Gagelmann et al. and I found that each LC spot refocuses as a discrete spot and no additional spots are generated (Figure 5).

A second explanation for the multiple LC forms is that some of them arise from proteolytic breakdown during the preparation. This explanation appears unlikely for the following reasons:

1. The LC forms have no detectable differences in molecular mass.

2. Proteolytic breakdown during the preparation of proteins from intact carotid arteries would require a protease active at

-196°C (boiling point of nitrogen), in 3% perchloric acid, 2% TCA, or 0.45% SDS.

3. Multiple LC forms are also obtained in aorta actomyosin despite the presence of three protease inhibitors, PMSF, leupeptin, and soybean trypsin inhibitor, during the preparation.

A third possibility is that some of the variants are not forms of the LC, but other polypeptides of very similar molecular mass and pI. This also is unlikely because:

1. The variants copurify when going from tissue to actomyosin to myosin (64). Also in the present work crude actomyosin contains the multiple forms.

2. The two unphosphorylated forms (spots 2 and 4) are phosphorylated in a similar manner when intact carotid arteries are stimulated (Table V) and when aorta homogenates or actomyosin are incubated with ATP (Figure 11). This argument is strengthened by the high substrate specificity of MLCK (p. 9).

3. The two unphosphorylated forms have very similar tryptic peptide maps (Figure 8) suggesting that they share extensive sequence homology.

It is therefore concluded that arterial smooth muscle LC consists of two isoforms. In their unphosphorylated forms, the isoforms have a proportion of 15 and 85% (Table II) and pIs 4.73 and 5.06 (Table III), respectively. Judging from their position in IEF gels, the two isoforms differ by two charges, the minor isoform

being more negatively charged. This difference may be generated by the following mechanisms:

1. Arterial smooth muscle LC is coded for by two different genes.

2. A single LC gene gives rise to two mRNAs through differential splicing of the primary transcript. Such mechanism of gene regulation that can produce protein isoforms has been described for several proteins including skeletal muscle myosin light chains (105). In both cases one might have two gene products with a difference in their number of acidic or basic amino acid residues that would result in a net difference of two charges.

3. A single LC gene codes for a single polypeptide which acquires or loses two charges by post-translational modifications that are not quantitative. Such modifications include deamidation of asparagine and glutamine and acylation of lysine and the terminal amino group.

The amino acid sequence of mammalian smooth muscle LC has not been reported to date, but the sequence of gizzard LC is known (25). Gizzard LC contains 23 lysine and arginine residues and is hydrolyzed by trypsin at 19 of these sites. Two of the 20 tryptic peptides produced are identical, leaving only 19 different peptides. Therefore the 16 ninhydrin-positive spots obtained on the peptide map of the major isoform of porcine arterial LC (Figure 8) may be considered satisfactory.

The tryptic peptide maps of the two isoforms differ in that spot 2 has two peptides that spot 4 does not have, and spot 4 has four peptides that spot 2 does not have. This difference can be explained if spot 4 has two arginine or lysine residues more than spot 2. This would present trypsin with two additional cleavage sites and might generate two more peptides. When one cleavage site in spot 4 does not exist in spot 2, two tryptic peptides of spot 4 are replaced by one peptide in spot 2, and when two cleavage sites in spot 4 do not exist in spot 2, four peptides of spot 4 may be replaced by two peptides in spot 2. The above hypothesis also explains the difference of -2 charges between spot 2 and 4, provided that the two arginine or lysine residues of spot 4 are replaced by two neutral amino acids in spot 2, or that two lysine residues of spot 4 are acylated.

It is not clear whether the existence of two LC isoforms in arterial smooth muscle is of any physiological importance. As mentioned above, the two isoforms have similar phosphorylation patterns. Since each myosin molecule contains two LC subunits, there are three possible myosin populations differing in LC composition: Two containing the same LC isoform at both positions and a mixed one. The fact that one isoform exists in excess of the other suggests that at least two different populations exist, one necessarily containing the major isoform at both positions. It would be interesting to separate these myosins and compare their properties, although this requires a system powerful enough to

detect subtle differences in the LC subunits within a 500-kDa molecule. Alternatively, one might reconstitute myosin from purified heavy chains, 17-kDa light chains, and LC isoforms.

Based on [^{32}P]phosphate analysis of its multiple electrophoretic variants, arterial smooth muscle LC was shown to be diphosphorylated in intact carotid arteries and triphosphorylated in aorta actomyosin incubated with ATP. Accordingly, the model outlined in Table VIII is proposed. The model summarizes the identification of the LC spots and shows (through the direction of the arrows) how arterial LC may display from two up to six discrete spots. Completely dephosphorylated LC consists of two spots (4 and 2) corresponding to two unphosphorylated isoforms. Homogenized tissue from freshly slaughtered animals contains a detectable amount of monophosphorylated major isoform (spot 3, Table II), and intact carotid arteries frozen at resting tension or after stimulation contain in addition monophosphorylated minor isoform (spot 1) and diphosphorylated major isoform in spot 2. The same four spots are detected in aorta homogenates incubated with ATP. Incubation of crude aorta actomyosin with ATP results in the appearance of diphosphorylated minor isoform (spot 0), triphosphorylated major isoform in spot 1, and triphosphorylated minor isoform (spot -1). The two isoforms overlap partially in spots 1 and 2. A scheme similar to that of Table VIII was proposed by this laboratory to explain the multiple forms of LC in intact rat uterus (70).

TABLE VIII

COMPOSITION OF MYOSIN LIGHT CHAIN FORMS OF ARTERIAL SMOOTH MUSCLE[a]

Spot number	-1	0	1	2	3	4
Light chain forms			P_3LC^a ←	P_2LC^a ←	PLC^a ←	LC^a
	P_3LC^b ←	P_2LC^b ←	PLC^b ←	LC^b		

[a] LC^a, major LC isoform; LC^b, minor LC isoform; PLC, monophosphorylated LC; P_2LC, diphosphorylated LC; P_3LC, triphosphorylated LC.

Serine and threonine residues serve as phosphorylation sites in the LC. Monophosphorylated LC contains only or mainly Ser-P, in agreement with the finding that phosphorylation of gizzard LC at serine is much faster than phosphorylation at threonine (87). Diphosphorylated LC contains Ser-P and Thr-P, in agreement with other reports (82,83,85,86). My data did not allow assignment of the third phosphate group exclusively to serine or threonine. In intact carotid arteries the total amount of Ser-P in the LC is 11 to 12 times more than Thr-P and their ratio is not affected by stimulation with 100 mM KCl or 50 μM norepinephrine, although the phosphate content of the LC is. The ratio of the two phosphoamino acids decreases significantly to approximately 8 upon stretching the arteries to 1.7 times their resting length. Again, this ratio is not affected by stimulation with 100 mM KCl while stretching. These findings show the preferential stimulation of a kinase activity specific for threonine in response to mechanical treatment of the tissue, as opposed to the equal stimulation of the serine- and threonine-specific kinase activities in response to chemical treatment.

The mechanism of preferential stimulation of the threonine-specific kinase activity by stretch is not known. It is possible that stretch releases an activator of that activity or increases the interaction between LC and the kinase. In any event, the data suggest that LC phosphorylation at threonine may play a role in the response of arteries to mechanical stress.

The difference in LC phosphorylation pattern between aorta homogenates and crude actomyosin (Figure 11) is remarkable. Particularly striking is the high degree of diphosphorylation and the appearance of triphosphorylated LC in actomyosin incubated with ATP. The time course of phosphorylation shows that the conversion of monophosphorylated to diphosphorylated to triphosphorylated LC is a random rather than sequential process. These findings establish the presence of kinases[1] able to di- and triphosphorylate the LC in arterial smooth muscle. These kinases remain in the crude actomyosin preparation and cause much higher phosphorylation than in intact tissue or in tissue homogenate. Why this happens requires further investigation. One possibility is that the kinases in question are prevented from interacting with the LC in the intact tissue or in the tissue homogenate by elements that are removed during the isolation of actomyosin. It must be noted, however, that such hindrance cannot be attributed to the conformation of myosin, since the phosphorylation of actomyosin was carried out at low ionic strength, at which myosin retains its native filamentous form. Moreover, the diphosphorylation of LC upon incubation of gizzard myosin with MLCK and ATP was achieved at low ionic strength (84,85), whereas no diphosphorylation was detected at high ionic strength (85). Another explanation of the high diphosphorylation and the

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[1] Plural is used because two protein kinases have been shown to diphosphorylate the LC thus far. It is understood, however, that there might be only one kinase capable of LC di- and triphosphorylation in arterial smooth muscle.

triphosphorylation of LC in actomyosin is that the respective kinases are activated or, reversely, an inhibitory factor is removed or inactivated during the preparation. Interestingly, Cole et al. (83) found that gizzard myosin which was collected by centrifugation immediately after precipitation with $MgCl_2$ and was incubated with MLCK and ATP, had only a trace of diphosphorylated LC, whereas myosin that was left to settle in the cold overnight before collection and incubation with ATP displayed 30-40% diphosphorylated LC. Although different, my method of actomyosin preparation also included overnight precipitation at 4°C (p. 33). In any case, there appears to be a regulatory mechanism that maintains the LC phosphorylation level well below 1 mol/mol in the intact tissue, even after maximal stimulation. Deficiencies of this mechanism might allow abnormally high levels of LC phosphorylation and might lead to potential pathological situations.

As already discussed (section II.G), MLCK and protein kinase C can phosphorylate monophosphorylated gizzard LC at threonine with opposite effects on the actin-activated MgATPase activity of myosin. My data provide no information about the nature of the kinases catalyzing the di- and triphosphorylation of arterial LC or about their effect on the properties of myosin. Protein kinase C is certainly a candidate, since it is present in aorta (106), it is activated by Ca^{2+}, and since the phospholipids required for its activation may become available through homogenization of the tissue or through stretching, if the latter causes some membrane

disintegration. Consideration of MLCK as a candidate for diphosphorylation contradicts the fact that only high levels of the enzyme were able to diphosphorylate gizzard LC (84,85). Although MLCK copurifies with actomyosin in 0.6 M KCl (23,24), it seems unlikely that an actomyosin preparation would be more enriched in MLCK than in myosin, i.e. have a higher enzyme to substrate ratio than the tissue homogenate. Moreover, LC diphosphorylation by MLCK was suppressed at Mg^{2+} concentrations higher than 1 mM (85). My phosphorylation system contained 5 mM Mg^{2+}. Furthermore, I also have to account for triphosphorylation. There are two reports on LC triphosphorylation in the literature: in whole myosin, by incubation with high amount of MLCK followed by protein kinase C (87), and in isolated LC, by protein kinase C alone (82). Thus, the identity of the endogenous kinases responsible for multiple phosphorylation of the LC has not been established as yet.

In conclusion, the main findings of the research presented herein are the following:

1. The multiple electrophoretic variants of arterial smooth muscle LC are not the result of experimental artifact, but genuine characteristics of smooth muscle myosin.

2. The LC consists of two isoforms with similar tryptic peptide maps and phosphorylation patterns.

3. The LC can be diphosphorylated to a small degree in intact carotid arteries. The main phosphorylation site is serine and the second phosphorylation site is threonine.

4. The molar ratio of Ser-P to Thr-P in intact carotid arteries is independent of chemical treatment, but it decreases significantly upon stretching.

5. The LC pattern is very different between aorta homogenate and crude actomyosin incubated with ATP in the presence of Ca^{2+} and Mg^{2+} at low ionic strength. Protein kinases present in the actomyosin preparation greatly increase the level of diphosphorylation and also triphosphorylate the LC.

These findings point to several directions for future research. Of prime interest is the identity of the endogenous protein kinases catalyzing the di- and triphosphorylation of LC. This can be probed by separating the protein components of crude actomyosin chromatographically, assaying for LC kinase activity, and characterizing the kinase fractions. Another target of investigation may be the mechanism of activation of these kinases during the preparation of actomyosin. The starting point of this investigation should be to take the intermediate preparations between tissue homogenate and crude actomyosin (that is, low ionic strength pellet and high ionic strength extract), incubate them with Ca^{2+}, Mg^{2+}, and ATP, and examine the LC phosphorylation pattern. The results of this experiment may show which treatment is critical for activation of the kinases and may point to the mechanism of activation. This, in turn, may provide clues as to why these kinases do not phosphorylate the LC in the whole tissue. Finally, the physiological significance of multiple phosphorylation should be studied beginning with its

effect on the myosin MgATPase activity. As discussed earlier, the regulation of smooth muscle contractility is not fully understood. The accepted hypothesis of LC phosphorylation-mediated regulation is based on the assumption that LC can be monophosphorylated only. This assumption no longer holds true, and it will be of interest to find out whether multiple phosphorylation contributes to the regulation of smooth muscle activity.

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