Arterial smooth muscle myosin contains nonphosphorylated and phosphorylated light chains that appear as 4 spots on two-dimensional, Coomassie blue-stained gel electrophoretograms at the 20,000–molecular weight level (referred to as spots 4 through 1 in order of decreasing isoelectric points). Anti-light chain recognizes the proteins in all 4 light chain spots. Complete dephosphorylation of light chain in muscle homogenate, by inhibiting myosin light chain kinase and by adding phosphatase, leads to 2 spots on two-dimensional gel electrophoretograms; both spots are visible on immunoblots. Stimulation (K⁺ or stretch) of smooth muscle results in increased light chain phosphorylation. Autoradiography of the gel electrophoretograms reveals that radioactive components are contained in spots 3, 2, 1, and in an additional spot with lower isoelectric point, referred to as spot 0. Phosphoamino acid analysis shows that spots 3 and 1 contain phosphoserine, whereas spots 2 and 0 contain phosphoserine and phosphothreonine. Two-dimensional phosphopeptide mapping of the trypsin-digested proteins from spots 3 and 1 shows predominantly 2 peptides; whereas from spots 2 and 0, it shows 5 peptides. Sodium dodecyl sulfate gel electrophoresis of the phosphopeptides obtained with *Staphylococcus aureus* V8 digestion gives identical maps for spots 3 and 2, which are different from the identical maps of spots 1 and 0. The results suggest that arterial smooth muscle myosin contains 2 nonphosphorylated 20,000-dalton light chain isoforms with different amino acid sequences and that each isoform can be mono- and diphosphorylated. (Circulation Research 1987;61:898–903)

Myosin exhibits polymorphism; the various forms may reflect different biological activities. The structural variants are characteristic for a specific muscle in a specific species. Furthermore, changes in structure and in biological activities of the myosin accompany developmental and functional adaptation of the muscle. It has been shown that the ATPase activity of myosin is related to the speed of contraction. The percent distribution of cardiac isomyosins depends on the developmental stage and thyroid state of the animal. Though many studies have been performed with striated muscles (for review see Kamm and Stull17 and Bailin18), it is important to study the structural variants of this light chain. In this paper, the 20,000-dalton light chain in muscle homogenate, by inhibiting myosin light chain kinase and by adding phosphatase, leads to 2 spots on two-dimensional gel electrophoretograms; both spots are visible on immunoblots. Stimulation (K⁺ or stretch) of smooth muscle results in increased light chain phosphorylation. Autoradiography of the gel electrophoretograms reveals that radioactive components are contained in spots 3, 2, 1, and in an additional spot with lower isoelectric point, referred to as spot 0. Phosphoamino acid analysis shows that spots 3 and 1 contain phosphoserine, whereas spots 2 and 0 contain phosphoserine and phosphothreonine. Two-dimensional phosphopeptide mapping of the trypsin-digested proteins from spots 3 and 1 shows predominantly 2 peptides; whereas from spots 2 and 0, it shows 5 peptides. Sodium dodecyl sulfate gel electrophoresis of the phosphopeptides obtained with *Staphylococcus aureus* V8 digestion gives identical maps for spots 3 and 2, which are different from the identical maps of spots 1 and 0. The results suggest that arterial smooth muscle myosin contains 2 nonphosphorylated 20,000-dalton light chain isoforms with different amino acid sequences and that each isoform can be mono- and diphosphorylated. (Circulation Research 1987;61:898–903)

**Materials and Methods**

Carotid arteries were obtained from freshly slaughtered hogs. The muscle strips were dissected, incubated with [³²P]orthophosphate, frozen at resting state, or stimulated (contracted with K⁺ for 1 minute or stretched 1.7 times the resting length) to elicit maximal light chain phosphorylation (0.7 mol phosphate/mol light chain), and then frozen as described previously.10 The extracts were extracted, then separated by two-dimensional gel electrophoresis using isoelectric focusing in the first dimension and sodium dodecyl sulfate (SDS) polyacrylamide gel in the second dimension.10,11 Stain and radioactivity distributions were measured and mol phosphate/mol light chain was calculated as described previously.12

**Dephosphorylation of Myosin Light Chain**

Crude active phosphatase was prepared from 2 g of arterial smooth muscle. The muscle was homogenized in 1.5 ml solution containing 150 mM NaCl, 1 mM EGTA, 20 mM Tris-HCl (pH 7.2), 1 mM DTT, 15 mM phenylmethylsulphonylfluoride, 0.1 mg/ml leupeptin, and 0.2 mg/ml soybean trypsin inhibitor. After centrifugation, β-mercaptoethanol was added to the supernatant to 1.6% (vol/vol). On freezing and thawing, it was centrifuged, and the supernatant was used as crude phosphatase.19 For the dephosphorylation of the light chain, about 10 g of frozen and pulverized arterial muscle powder was stirred for 3 hours at 22 °C in 25 ml solution containing 150 mM NaCl, 1 mM EGTA, 20 mM Tris-HCl (pH 7.2), 1 mM DTT, 15 mM phenylmethylsulphonylfluoride, 0.1 mg/ml leupeptin, 0.2 mg/ml soybean trypsin inhibitor, and the 1.5 ml crude phosphatase. The reaction was stopped by adding to the
suspension trichloroacetic acid to 10%. After centrifugation, the pellet was solubilized as described previously.10,11

**Immunological Studies**

The 20,000-dalton spots were transferred from the two-dimensional gel by electroblotting to a nitrocellulose membrane, the vacant protein-binding sites of the membrane were blocked with bovine serum albumin, and then the membrane was incubated overnight with antiserum from a rabbit immunized with the 20,000-dalton light chain purified from bovine tracheal smooth muscle. After washing out the nonspecifically bound first antibody, the membrane was incubated for 6 hours with goat anti-rabbit IgG conjugated to horseradish peroxidase. The membrane was washed exhaustively to remove the nonspecifically bound second antibody. The formation of immunocomplexes was visualized by using 4-chloro-1-naphthol as a peroxidase substrate.13,20

**Phosphopeptide Mapping**

Two-dimensional gel electrophoresis was performed with extracts from stimulated muscles containing maximally phosphorylated light chains. The individual radioactive spots, localized by autoradiography, were dissected from the gels, and the proteins were eluted with 50 mM NH₄HCO₃. Depending on the radioactivity incorporated into the proteins of the individual spots, up to 8 gel pieces were pooled before elution. After centrifugation and exhaustive dialysis, the samples were freeze-dried. The freeze-dried powders were dissolved in 0.2 ml of 10 mM NH₄HCO₃ and digested at 37° C by 5 repeated additions of 5 µl of 1 mg/ml trypsin (dissolved in 1 mM HCl, TPCK-treated, purchased from Sigma Chemical Co., St. Louis, Mo.) at 45-minute intervals; the digestion was continued for a total of 6 hours. The samples were centrifuged, and the supernatants were freeze-dried. Electrophoresis was performed in acetic acid/formic acid/water (150: 50: 800 vol/vol) at pH 1.9 at 300 V for 90 minutes on Kodak Chromagram 13255 cellulose sheet (first dimension), followed by ascending chromatography (second dimension) in butanol/pyridine/acetic acid/water (15: 10: 3: 12 vol/vol) for 3 hours. This procedure eliminated any radioactive material remaining at the origin on the cellulose sheets and, hence, resulted in reproducible maps.

To ensure that the procedure of trypsin digestion was appropriate, the amount of trypsin was increased to 200 µg in additional experiments, and the time was extended to 16 hours. These changes did not alter the peptide maps.

*Staphylococcus aureus* V8 protease (specifically cleaving peptide bonds on the COOH terminal side of glutamic acid, purchased from ICN ImmunoBiologicals, Lisle, Ill.) was used to obtain a peptide digest different from that obtained with trypsin. The eluted, dialyzed, and clarified samples were freeze-dried, then dissolved in 0.125 M Tris-HCl (pH 6.8), 0.032 M DTT, 0.1% SDS, 20% glycerol, 0.0001% bromphenol blue.

The samples were electrophoresed on a slab gel, consisting of a 9 cm long 15% polyacrylamide gel in 0.1% SDS, 0.37 M Tris-HCl (pH 8.8), and of a 5 cm long 5% polyacrylamide gel in 0.1% SDS and 0.125 M Tris-HCl (pH 6.8) in which the 10-well comb was inserted. The samples in the wells were overlayed first with 10 µg *S. aureus* V8 protease dissolved in 0.125 M Tris-HCl (pH 6.8), 0.032 M DTT, 0.1% SDS, 10% glycerol, 0.0001% bromphenol blue, then with 0.125 M Tris-HCl (pH 6.8), 0.032 M DTT, 0.1% SDS. Electrophoresis was carried out at 20 mA for about 2 hours, and when the bromphenol blue reached the bottom of the stacking gel, the current was turned off for 30 minutes. Electrophoresis was continued in the resolving gel for about 3 hours.21

**Phosphoamino Acid Analysis**

Two-dimensional gel electrophoresis was performed with proteins from stimulated muscles. The protein from each radioactive spot of the gel was eluted, freeze-dried, dissolved in 6 M HCl, and partially hydrolyzed at 110° C for 1 hour. The hydrolysate was evaporated to dryness, dissolved in water, mixed with unlabelled phosphoamino acid markers, and electrophoresed at pH 1.9 on Kodak Chromagram 13255 cellulose sheet. The dried electrophoretograms were ninhydrin stained and the [³²P]phosphoamino acids were identified by autoradiography.22

Alternatively, peptide mapping was performed with the protein eluted from the gel; then, each peptide was scraped separately from the cellulose sheets. The peptides from 2–12 spots were pooled. After elution and centrifugation, the peptides were freeze-dried, and the above procedure of phosphoamino acid analysis was followed.

**Results**

**Immunologic Studies**

The phosphorylatable 20,000-dalton light chain was resolved into 4 spots (numbered 4 to 1 in order of decreasing isoelectric points) on Coomassie blue-stained electrophoretogram (Figure 1, top panel). Autoradiography revealed that spots 3, 2, and 1 were radioactive. An additional spot toward lower pH, not visible on the stained electrophoretogram, was radioactive and is labelled spot 0 (Figure 1, middle panel). Spots 4, 3, 2, and 1 are seen on the immunoblot (Figure 1, bottom panel). The percent stain distribution in the 4 spots of the electrophoretogram and of the immunoblot is the same. The finding that the anti-light chain recognizes the proteins in all four spots from the gel provides evidence that all four spots contain myosin light chain and none of the spots originate from contaminants.

Myosin light chain in living arterial smooth muscle exists in phosphorylated and nonphosphorylated forms. To determine the number of the nonphosphorylated isoforms, the light chains in the extract of the muscle powder were completely dephosphorylated. The nonphosphorylated light chains exhibited two spots on the Coomassie blue-stained electrophoreto-


Stain and Radioactivity Distributions in the Light Chain Spots

Whereas the four-spot pattern on two-dimensional Coomassie blue-stained electrophoretogram is characteristic for the 20,000-dalton light chain in living smooth muscle (see Figure 1, top panel), the stain and radioactivity distributions in the light chain spots depend on the functional state of the muscle. Table 1 presents the measured stain and radioactivity distributions and the estimated mol phosphate/mol light chain in each spot when the muscle was at rest or contracted with K+ for 1 minute. It has been previously shown that during K+ stimulation the level of light chain phosphorylation increases over the level in resting muscle, but different phosphorylation values were obtained from densitometric scanning of the separated radioactive and nonradioactive spots on electrophoreograms (S values) and from determination of the incorporated radioactivity (P values). The discrepancy can be easily explained based on the results presented here. The estimated mol phosphate/mol light chain is close to 1 in spots 3 and 1, but in spot 2, it is 0.3 in the resting muscle and 1.4 in the stimulated muscle (Table 1). In the resting muscle, the estimated mol phosphate/mol light chain in spot 2 is low (<1) because of the presence of nonphosphorylated isoform; as the muscle is stimulated, part of the nonphosphorylated isoform becomes phosphorylated; hence, the amount of the nonphosphorylated component decreases in spot 2. Furthermore, upon stimulation of the muscle, the light chains become monophosphorylated and diphosphorylated (see following sections of "Phosphoamino Acid Analysis" and "Phos-

<table>
<thead>
<tr>
<th>Spot</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stain distribution</td>
<td>ND</td>
<td>3 ± 1</td>
<td>11 ± 2</td>
<td>22 ± 3</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>Radioactivity distribution</td>
<td>3 ± 1</td>
<td>11 ± 2</td>
<td>14 ± 2</td>
<td>72 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>Mol phosphate/mol light chain</td>
<td>ND</td>
<td>1.0</td>
<td>0.3</td>
<td>0.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 1. Stain and Radioactivity Distributions of the Phosphorylatable Light Chain in Spots on Two-Dimensional Electrophorectogram at the 20,000-Dalton Level

S, Sum of the fractional staining intensities in the spots exhibiting radioactivity (spots 1, 2, and 3). P, Actual phosphorylation in mol phosphate/mol light chain based on measuring the radioactivity incorporated into light chain. Values are mean ± SD, n = 6.

ND, Not determined.
Phosphoamino acid analysis of individual light chain spots. A, Ninhydrin stain pattern of unlabelled marker phosphoamino acids; B, autoradiograms obtained with the samples; pep-P, phosphopeptide.

The relation between the S and P values was determined by plotting the data pairs and fitting a straight line according to the least-squares method:

$$P = \frac{S - 0.20}{0.72}$$

where $P$ is the actual phosphorylation in mol phosphate/mol light chain based on measuring the incorporated radioactivity and $S$ is the sum of the fractional staining intensities in the spots exhibiting radioactivity, spots 3, 2, and 1.

**Phosphoamino Acid Analysis**

Figure 3 shows the phosphoamino acid analysis of the individual light chain spots. Spots 3 and 1 contain phosphoserine, whereas spots 2 and 0 contain phosphoserine and phosphothreonine. Thus, the two non-phosphorylated isoforms of spots 4 and 2 can be monophosphorylated at a serine site and constitute spots 3 and 1, and they can be diphosphorylated at serine and threonine sites, which are located in spots 2 and 0. The results were the same for K$^+$- or stretch-stimulated muscles.

**Phosphopeptide Mapping**

Figure 4 shows the two-dimensional phosphopeptide maps of the trypsin-digested proteins eluted separately from spots 3, 2, 1, and 0. Spots 3 and 1 contained predominantly 2 peptides, A and B, which were separated only in the second dimension. It should be emphasized that these peptides were well separated and the radioactivity in peptide A was 2–2.5 times higher than that in peptide B. A third peptide, E, was slightly visible; its radioactivity was less than 5% of the total. In addition to these peptides, spots 2 and 0 contained two more peptides, C and D, which were also separated only in the second dimension. The percent radioactivity of peptide E in spots 2 and 0 was significantly increased compared to that in spots 3 and 1. The same peptide maps as shown in Figure 4 were obtained from either K$^+$- or stretch-stimulated muscles.

Phosphoamino acid analysis showed that peptides A and B contained phosphoserine and that peptides C and D contained phosphothreonine. These results are consistent with the conclusion that spots 3 and 1 are the monophosphorylated forms of the two isoforms and that spots 2 and 0 are derived from the monophosphorylated light chains by a second-site phosphorylation. Thus, spot 0 contains diphosphorylated light chain, whereas spot 2 consists of the nonphosphorylated form of one of the isoforms and of the diphosphorylated form of the other isoform.

Peptide E contained phosphoserine. The amino acid analysis of the peptides is in accordance with that of the light chain spots. Namely, peptides A, B, and E containing phosphoserine are in spots 3 and 1, which showed phosphoserine. Peptides A, B, and E containing phosphoserine and peptides C and D containing phosphothreonine are in spots 2 and 0, which showed phosphoserine and phosphothreonine (Figure 3).

The different nature of the two isoforms was manifested by SDS electrophoresis of the radioactive phosphopeptides obtained by S. aureus V8 digestion of the proteins from the individual light chain spots. Figure 5 shows diffuse bands, which migrate as a single entity for spots 3 and 2, whereas 2 separate bands are seen for spots 1 and 0. Though the autoradiograms exhibit wide bands, the difference shown in Figure 5 was reproduced in 12 separate experiments.

**Discussion**

The 20,000-dalton myosin light chain exists in phosphorylated and nonphosphorylated forms in the intact arterial smooth muscle. Whereas contraction is coupled with increased light chain phosphorylation, the resting or pharmacologically relaxed muscle still contains phosphorylated light chain. To obtain completely dephosphorylated light chain, it is necessary to inhibit myosin light chain kinase. To shorten the
FIGURE 5. Autoradiograms of phosphopeptide maps obtained with Staphylococcus aureus V8 digested proteins from each individual light chain spot. Phosphopeptides were subjected to SDS polyacrylamide gel electrophoresis. LC, undigested light chain.

time of dephosphorylation and to avoid degradation of the light chain during the dephosphorylation process, active phosphatase and protease inhibitors were added to the muscle homogenate. Such completely dephosphorylated light chain exhibited two spots on two-dimensional Coomassie blue-stained gel electrophoretograms, and polyclonal anti-light chain recognized the proteins in both spots (Figure 2). These findings suggest that the 20,000-dalton myosin light chain contains two distinct nonphosphorylated isoforms. Anti-light chain also recognized their phosphorylated forms (Figure 1).

Previous work from this laboratory analyzed the tryptic peptide maps of light chains from spots 4 and 2 by ninhydrin staining. The results showed that the light chains in spots 4 and 2 are related, but they are distinct entities. The data of present work with S. aureus V8 digestion of the 32P-labelled light chain also show a difference between the two isoforms (Figure 5). From LC*, only one radioactive band is generated on phosphorylation, whereas LC gives rise to two bands. This indicates a difference in the location of glutamic acid residues in the primary sequence of the isoforms.

The data of phosphoamino acid analysis (Figure 3) indicate that the light chains are phosphorylated in vivo at both serine and threonine residues. The two-dimensional tryptic phosphopeptide maps (Figure 4) suggest that the light chains are phosphorylated at 5 distinct sites involving three different serine and two different threonine residues. The same phosphopeptides (A, B, and E, Figure 4) are seen on the maps from spots 3 and 1, each containing phosphoryserine. The intensity of the peptide spots, as well as the radioactivity eluted from the spots, clearly indicate that the different serine residues in the muscle are phosphorylated at different rates. Peptide A is the primary site for serine phosphorylation, whereas in peptide E, the phosphorylation of serine is low. Peptides A, B, and E are also constituents of the light chain forms that are localized in spots 2 and 0. Furthermore, these spots include two additional peptides, C and D, containing phosphothreonine (Figure 3).

It is of interest to compare our peptide map (Figure 4) with that of Bengur et al obtained from turkey gizzard light chain phosphorylated with protein kinase C in vitro (see their Figure 1B). Our peptide E is positioned similarly to peptide 1 of Bengur et al. Furthermore, both peptides contain phosphoserine. This suggests that protein kinase C phosphorylates a serine residue in vivo.

Peptide 1 of Bengur et al is a doublet that was shown by the authors to contain the same amino acid sequence; thus, the doublet corresponds to a single phosphopeptide. This finding raises the question whether peptides A and B or peptides C and D could also represent one phosphopeptide. However, unlike peptide 1 of Bengur et al, peptide A is well separated from B, and peptide C is well separated from D (Figure 4). Furthermore, increasing the amount of trypsin by 8 times and the digestion time by 3 times did not change the peptide maps. Finally, S. aureus V8 protease digestion yielded 2 different peptides from spot 1 (Figure 5), which predominantly contains peptides A and B (Figure 4), suggesting that peptides A and B contain different serine residues. These considerations support the idea that the 5 phosphopeptides separated in this work represent 5 different sites available for phosphorylation.

From our present data, one can delineate the phosphorylation events that take place in the light chain structure during stimulation of the muscle. These are summarized in Table 2. It is assumed that the major isoform, LC* in spot 4, upon phosphorylation on sites either A, B, or E yields the monophosphorylated PLC in spot 3. Similarly, the minor isoform, LC in spot 2, upon phosphorylation on sites either A, B, or E yields the monophosphorylated PLC in spot 1. Furthermore, a second-site phosphorylation of PLC on sites either C or D results in diphosphorylated P2LC migrating from spot 3 to spot 2. Similarly, a second-site phosphorylation of PLC at sites C or D results in diphosphorylated P2LC migrating from spot 1 to spot 0.

The physiological role of the multiple-site light chain phosphorylation is not known. In vitro studies

Table 2. Composition of the Phosphorylatable Myosin Light Chain in Spots on Two-Dimensional Electrophoretogram at the 20,000-Dalton Level

<table>
<thead>
<tr>
<th>Spot</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.45</td>
<td>4.60</td>
<td>4.75</td>
<td>4.90</td>
<td>5.05</td>
</tr>
<tr>
<td>Phosphopeptides</td>
<td>A,B,C,D,E</td>
<td>A,B,E</td>
<td>A,B,C,D,E</td>
<td>A,B,E</td>
<td></td>
</tr>
</tbody>
</table>

| Light chain isoforms |              |              |
|----------------------|--------------|
| Nonphosphorylated    | LCb          | LCa          |
| Monophosphorylated on serine residues | PLCb | PLCa |
| Diphotosphorylated on serine residues, and threonine residues | P2LCb | P2LCa |
revealed multiple-site phosphorylation (different serine and threonine residues) in isolated gizzard light chain by myosin light chain kinase and protein kinase C.25,26 The primary phosphorylation site in arterial smooth muscle corresponds to peptide C, and the primary enzyme is the myosin light chain kinase. The secondary phosphorylation site in vivo corresponds to peptide C, and either the light chain kinase or protein kinase C may be involved.25,26 Double phosphorylation of turkey gizzard myosin light chain was demonstrated by Ikebe et al.23 in vitro, involving myosin light chain kinase and two adjacent residues, serine 19 and threonine 18. Because we did not detect both phosphoserine and phosphothreonine residues within a single peptide, it appears that myosin light chain kinase is not responsible for a second-site phosphorylation of arterial myosin light chain on a threonine residue. Alternatively, if myosin light chain kinase is involved in threonine phosphorylation in vivo, there must be a difference in the primary sequence between turkey gizzard and porcine carotid light chains.

One may speculate on the significance of the 5 phosphorylation sites. Thus, theoretically if all 5 sites would be phosphorylated at once, this would result in an increase of 9 negative charges (at physiological pH, each phosphorylated serine or threonine residue yields 1.8 negative charges) in the light chain polypeptide, and this certainly would result in a major conformational change in the protein structure. It is unlikely that such an extreme modification takes place under physiological conditions. However, limited conformational changes induced by restricted phosphorylation are likely. These events may control the precise tuning of the cross-bridge interaction with the actin filaments during arterial smooth muscle contraction.

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We are indebted to Dr. James T. Stull, who generously donated the anti-light chain. We express our appreciation to our former collaborators, Drs. John T. Barron, Sándor Csabina, and Vassilis Mougiou for valuable discussions. We thank Kathryn Peregrine for dedicated assistance and Donna M. Lattyak for careful typing of the manuscript.

References

6. Rovner AS, Thompson MM, Murphy RA: Two different heavy chains are found in smooth muscle myosin. Am J Physiol 1986;250:C661–C670

KEY WORDS • myosin light chain isoforms • light chain phosphorylation • phosphopeptide mapping • arterial smooth muscle
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F Erdödi, M Bárány and K Bárány

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