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Isoforms of the phosphorylatable myosin light chain in arterial smooth muscle

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Two isoforms of the phosphorylatable myosin light chain of arterial smooth muscle have been identified, at proportions of 15 and 85%. The isoforms have similar tryptic peptide maps and can be mono-, di- and triphosphorylated. In intact or homogenized muscle, monophosphorylation and, to a small degree, diphosphorylation occur, whereas in isolated actomyosin diphosphorylation and triphosphorylation are manifested. Serine and threonine residues are phosphorylated in all three systems, but the ratio of phosphothreonine to phosphoserine is much higher in actomyosin than in muscle.

In the effort to evaluate the role of myosin light-chain phosphorylation in the regulation of smooth muscle contraction, the technique of two-dimensional gel electrophoresis has been instrumental to many investigators. When this technique was used with arterial and uterine smooth muscle proteins, the 20-kDa myosin light chain was resolved not only in its phosphorylated and unphosphorylated form, but in two additional spots of lower isoelectric points [1–6]. The nature of these spots has been debated. One possibility is that they originate from the two main light-chain spots as the result of artifactual charge modification [1,4], but data have also been presented against this hypothesis [3]. Another possibility, which is supported by studies with isolated proteins [7–12], is multiple phosphorylation of the light chain. In this study we show that porcine arterial smooth muscle contains two light-chain isoforms, which can be mono-, di- and triphosphorylated. Furthermore, we show major differences in light-chain phosphorylation pattern between intact or homogenized muscle and isolated actomyosin. A preliminary report on this work has been presented [13].

The following experiments indicate that the four spots of arterial light chain observable on two-dimensional gels are not artifacts: (1) preelectrophoresis of the gels in the presence of 1 mM sodium thioglycolate, which has been reported to eliminate charge modification [4], had no effect on the light-chain spots under our conditions [2]; (2) two-dimensional IEF [3] showed all light-chain spots arranged in a diagonal pattern and no off-diagonal spots generated below any original spot; (3) the same gel electrophoretic procedure which gives four light-chain spots in arterial and uterine smooth muscle shows only two spots of the phosphorylatable myosin light chain in skeletal muscle [14].

To identify the multiple light-chain spots (numbered *1–4* in order of increasing isoelectric point in this laboratory), we effected complete dephosphorylation of the light chain either by storing carotid arteries in glucose-free physiological salt

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Abbreviations: IEF, isoelectric focusing; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Ser-*P*, phosphoserine; Thr-*P*, phosphothreonine.

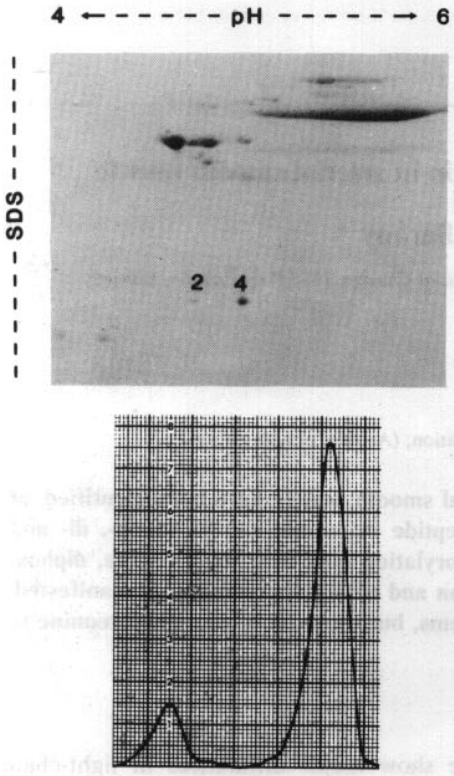

Fig. 1. Dephosphorylated light chain of arterial smooth muscle myosin. Carotid arteries were homogenized in 150 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.3), and were incubated at 25°C for 2 h. Proteins were analyzed by two-dimensional gel electrophoresis [2]. Top, Coomassie blue-stained gel. Spots *2* and *4* of the light chain are indicated. Bottom, densitometric tracing of the light-chain spots.

solution at 4°C for 3 days, or by incubating carotid artery or aorta homogenates with 1 mM EGTA for 2 h. When such preparations were analyzed by two-dimensional gel electrophoresis, only spots *2* (isoelectric point 4.73) and *4* (isoelectric point 5.06) appeared, at proportions of 15 and 85%, respectively (Fig. 1).

The relationship of these two unphosphorylated forms to each other was investigated by tryptic peptide mapping. Spots *2* and *4* were purified as follows: porcine aorta actomyosin, prepared as described [15], was denatured to release the light chains [16]; light chain was isolated from the total light chains by preparative SDS-polyacrylamide gel electrophoresis; and spots *2* and *4* were separated by preparative IEF of the light chain. Their tryptic peptide maps (Fig. 2) show the majority of the tryptic peptides (twelve) at identical positions. 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*. These results show that spots *2* and *4* are related, though distinct, entities, i.e. they are isoforms of the light chain. It should be noted that the total number of tryptic peptides is in good agreement with the nineteen different tryptic peptides obtained from gizzard light chain [17].

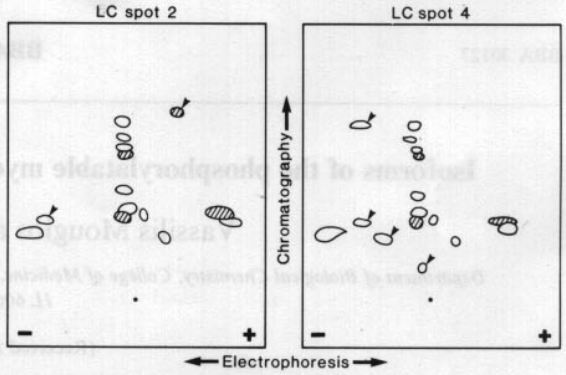

Fig. 2. Tryptic peptide maps of the unphosphorylated light-chain forms. The two unphosphorylated forms (spot *2* and spot *4*) were purified from porcine aorta and were digested with trypsin at an enzyme-to-substrate ratio of 1:100. Two-dimensional peptide mapping was performed on thin-layer cellulose sheets. Electrophoresis was in pyridine/acetic acid/water (1:10:189, v/v), and chromatography in 1-butanol/pyridine/acetic acid/water (15:10:3:12, v/v). Peptides were visualized with ninhydrin. 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 (●).

As shown before [2,18], upon stimulation of arterial muscle by KCl or norepinephrine or stretching, considerable phosphorylation of the light chain occurs. This is evidenced by a stain distribution of 5–8% in spot *1*, 11–16% in spot *2*, 46–56% in spot *3*, and 24–36% in spot *4*. Spot *1* is formed from spot *2*, and spot *3* is formed from spot *4*. Characteristic of the intact muscle is that the major changes in phosphorylation take place between spots *3* and *4*, whereas spots *1* and *2* remain minor components of the light chain.

An entirely different phosphorylation pattern was found when aorta actomyosin [15] was in-

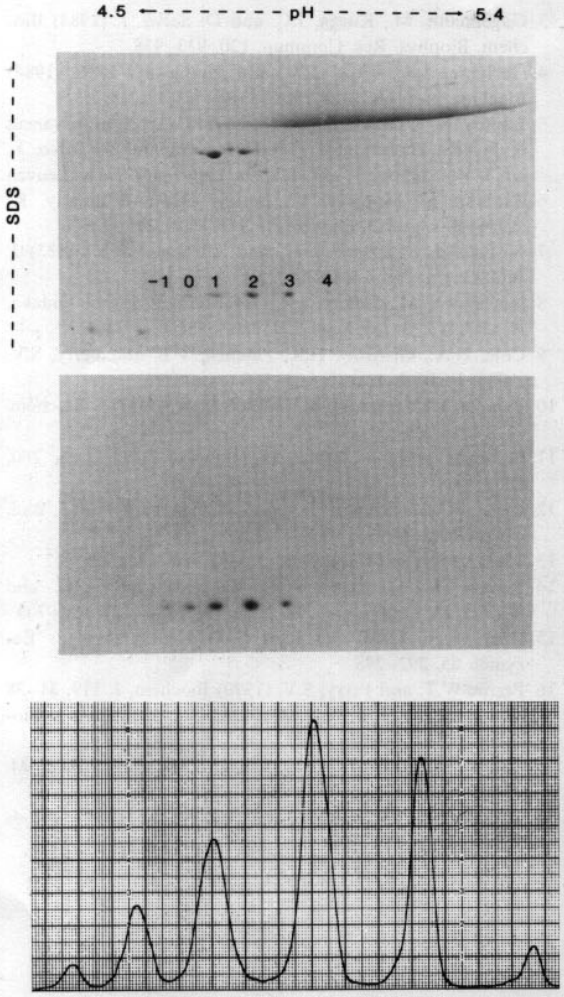

Fig. 3. Phosphorylation pattern of actomyosin. Aorta actomyosin was incubated in 35 mM KCl, 50 mM 4-morpholinepropanesulfonic acid, 1 mM dithiothreitol, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (pH 7.0) at 25°C for 10 min. Proteins were analyzed by two-dimensional gel electrophoresis. Top, Coomassie blue-stained gel. Spots *−1* to *4* of the light chain are indicated. Middle, autoradiogram of the gel. Bottom, densitometric tracing of the light-chain spots in the Coomassie blue-stained gel.

cubated with [γ-^{32}P]ATP (Fig. 3). In this system, spot *2* became the major light-chain spot. At the same time, two additional spots with lower isoelectric points than spot *1* appeared (Fig. 3, top). These spots will be referred to as spots *0* and *−1*. The distribution of staining intensity among spots *−1, 0, 1, 2, 3* and *4* at the peak of phosphorylation was 1, 6, 17, 42, 31 and 3%, respectively (bottom). Five of the six spots were radioactive (middle). The phosphate content of each spot was determined by dividing the mol of [^{32}P]phosphate by the mol of light chain in each spot. This determination was not possible for spot *−1* because of its low staining intensity. The mean ± S.D. values for the remaining five spots were 2.27 ± 0.43, 2.70 ± 0.24, 1.70 ± 0.17, 0.92 ± 0.10 and 0.10 ± 0.17 mol [^{32}P]phosphate/mol light chain (n = 14), respectively. The stoichiometry suggested by these data is 2, 3, 2, 1 and 0, respectively.

The difference in light-chain phosphorylation between intact muscle and actomyosin is not limited to staining patterns only. The phosphate content of spots *1, 2, 3* and *4* in intact carotid arteries labeled with $^{32}P_i$ [18] was found to be 1.19 ± 0.44, 0.65 ± 0.47, 0.83 ± 0.29 and 0.04 ± 0.04 mol [^{32}P]phosphate/mol light chain (n = 66), respectively. The low phosphate contents of spots *1* and *2*, as compared to the respective values in actomyosin (2.70 and 1.70), suggest that no detectable triphosphorylation and only limited diphosphorylation occurs in intact arteries.

A characteristic difference between intact muscle and actomyosin was also found in the phosphoamino acid content of the light chain. Phosphoamino acid analysis [19] of the radioactive light-chain spots in ^{32}P-labeled carotid arteries showed that spots *1* and *3* contained Ser-*P*, whereas spot *2* contained both Ser-*P* and Thr-*P* in comparable amounts. In actomyosin, spots *−1, 0, 1* and *2* contained Ser-*P* and Thr-*P* in comparable amounts, and spot *3* contained Ser-*P* and a small amount of Thr-*P*. Consequently, the molar ratio of Ser-*P* to Thr-*P* was 11 in intact arteries but only 1.3 in actomyosin.

It is of interest to note that the differences between intact muscle and actomyosin are not based on the integrity of the muscle, because aorta homogenates incubated with ATP under the same conditions as actomyosin displayed a light-chain phosphorylation pattern very similar to that of stimulated intact arteries. Therefore, the changes observed in actomyosin must be attributed to the removal of a factor(s) which is present in both intact and homogenized muscle.

Based on the above data, the following scheme is proposed, depicting the phosphorylation pattern

of the two light-chain isoforms:

Spot number	−1	0	1	2	3	4
LC forms	$P_3LC^b \leftarrow P_2LC^b \leftarrow PLC^b \leftarrow LC^b$		$P_3LC^a \leftarrow P_2LC^a \leftarrow PLC^a \leftarrow LC^a$			

where LC^a and LC^b are the two unphosphorylated isoforms, and PLC, P_2LC, and P_3LC are mono-, di- and triphosphorylated light chain, respectively. In intact or homogenized muscle, monophosphorylation and a low level of diphosphorylation take place, whereas in actomyosin both a high level of diphosphorylation and triphosphorylation occur. A scheme similar to the above has been proposed by this laboratory to explain the multiple forms of the light chain in intact rat uterus [5].

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