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STRETCH ACTIVATES MYOSIN LIGHT CHAIN KINASE IN ARTERIAL SMOOTH MUSCLE

Kate Bárány,[1] Anikó Rokolya[1] and Michael Bárány[2]

[1]Departments of Physiology and Biophysics and [2]Biochemistry,
College of Medicine, University of Illinois, Chicago, IL 60612

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SUMMARY: Stretching of porcine carotid arterial muscle increased the phosphorylation of the 20 kDa myosin light chain from 0.23 to 0.68 mol [^{32}P]phosphate/mol light chain, whereas stretching of phorbol dibutyrate treated muscle increased the phosphorylation from 0.30 to 0.91 mol/mol. Two-dimensional gel electrophoresis followed by two-dimensional tryptic phosphopeptide mapping was used to identify the enzyme involved in the stretch-induced phosphorylation. Quantitation of the [^{32}P]phosphate content of the peptides revealed considerable light chain phosphorylation by protein kinase C only in the phorbol dibutyrate treated arterial muscle, whereas most of the light chain phosphorylation was attributable to myosin light chain kinase. Upon stretch of either the untreated or treated muscle, the total increment in [^{32}P]phosphate incorporation into the light chain could be accounted for by peptides characteristic for myosin light chain kinase catalyzed phosphorylation, demonstrating that the stretch-induced phosphorylation is caused by this enzyme exclusively.

Since the proposal of Bayliss (1) that stretch is a stimulus for blood vessel constriction, numerous studies have dealt with the mechanism of stretch-induced contraction. We have shown that stretch induces phosphorylation of the 20 kDa myosin light chain (LC) in arterial smooth muscle (2) and that the extent of phosphorylation is a function of the applied stretch on the arterial strips, reaching a maximum at 1.7 times the resting length. When the fully stretched arteries were released, active tension developed without any exogenous stimulating agent (3). It is well accepted that specific phosphorylation of LC in intact smooth muscle is conferred by MLCK (4), but several authors have reported PKC catalyzed LC phosphorylation in muscles treated with PDBu

Abbreviations: LC, 20 kDa myosin light chain; MLCK, myosin light chain kinase; PKC, protein kinase C; PDBu, phorbol dibutyrate; PSS, physiological salt solution; 2D, two-dimensional.

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(5-8). Recent work of Laher, et al. (9-10) focused renewed attention on PKC which potentiated stretch-induced vascular tone. This was demonstrated with rabbit facial vein using staurosporine, a PKC inhibitor (9), and with rabbit basilar artery using phorbol 12-myristate 13-acetate, a PKC activator (10).

We described a detailed procedure for differentiation of LC phosphorylation by MLCK versus PKC (11): Two-dimensional tryptic phosphopeptide mapping yielded four peptides from LC upon MLCK catalyzed phosphorylation, referred to as A through D; phosphoserine residues were in peptides A and B, and phosphothreonine residues in peptides C and D. Two phosphopeptides were separated from LC upon PKC catalyzed phosphorylation, a phosphoserine residue in peptide E and a phosphothreonine residue in peptide F. We have used this procedure to assess PKC involvement in stretch-induced LC phosphorylation of porcine carotid arteries. Our data indicate that MLCK, not PKC, is the enzyme which produces this phosphorylation.

EXPERIMENTAL PROCEDURES

Carotid arteries were obtained from freshly slaughtered pigs. Muscle strips were dissected, mounted, resting tension was applied to simulate 100 mm Hg mean arterial pressure and the corresponding resting length was measured (3). The strips were incubated in PSS containing 2 mCi carrier-free [^{32}P]orthophosphate at 37°C for 90 min, washed 15 times with PSS for 30 min, stimulated and frozen in liquid nitrogen as described (3). PDBu stimulation was employed at 0.8 μM concentration in PSS for 1 hr. Stretch stimulation was induced by stretching the strip to 1.7 times the resting length within 20 sec followed by immediate freezing (2). The combined PDBu and stretched stimulations were elicited by first treating the muscle with PDBu for 1 hr, then stretching and immediate freezing.

The frozen muscle strips were pulverized to a powder by percussion using liquid nitrogen-chilled mortars and pestles in the cold room at 4°C. The frozen powder was immediately extracted with 3% perchloric acid and after centrifugation the supernatant was saved for determination of the specific radioactivity of [^{32}P]phosphocreatine, which was found to be equal to that of the specific radioactivity of [γ-^{32}P]phosphate of ATP. The residue of the centrifugation was washed three times with a solution containing 2% trichloroacetic acid and 5 mM KH_2PO_4 then solubilized in 0.25 M Na_2HPO_4 and 0.5% SDS. The solubilized proteins were dialyzed against 2000 volumes of 0.02% SDS and 1.0 mM $(NH_4)HCO_3$ at 25°C overnight. After clarification in the ultracentrifuge, LC was separated by 2D gel electrophoresis and LC phosphorylation was calculated as described (12).

Two-dimensional tryptic phosphopeptide mapping was performed on Kodak Chromagram 13255 cellulose sheets (11).

RESULTS

We have employed PDBu to activate PKC in the arterial muscle; at 0.8 μM concentration it elicits slowly developing contraction reaching in 1 hr about 50% of the maximal force produced by 100 mM K^+ (8). During 1 hr of PDBu treatment, 0.30 mol [^{32}P] phosphate is incorporated per mole LC in the muscle; when the PDBu-treated muscle is stretched, the incorporation is increased to 0.91 mol/mol (Table 1). Untreated resting muscles incorporate 0.23 mol [^{32}P]phosphate and, when stretched, 0.68 mol [^{32}P]phosphate (Table 1).

The difference in the incorporation between LC of PDBu-treated muscle and PDBu-treated and subsequently stretched muscle was analyzed by 2D gel electrophoresis (Figure 1). Four LC spots are seen in the stained gels (top) called Spots 1, 2, 3 and 4 from the acidic to the alkaline pH. Spot 4 has the highest staining intensity in the PDBu-treated muscle; this represents the unphosphorylated major LC isoform. In the PDBu-stretched muscle, Spot 3 has the highest staining intensity; this represents the monophosphorylated major isoform. In the corresponding autoradiograms of these gels (bottom), the PDBu-treated muscle shows three radioactive spots which correspond to staining Spots 1, 2 and 3. The autoradiogram of LC from the PDBu-stretched muscle shows four spots which correspond to staining Spots 1, 2 3 and to a spot not observable by staining but only by radioactivity, called Spot 0. This radioactive spot could also be detected in the gel of PDBu-treated muscle when the corresponding area was excised,

Table 1. Effect of PDBu and stretch on myosin light chain phosphorylation in arterial muscles

Treatment	Mol [^{32}P]phosphate/mol LC	n
PDBu	0.30 ± 0.07	9
PDBu and stretch	0.91 ± 0.13	12
None	0.23 ± 0.08	6
Stretch	0.68 ± 0.11	8

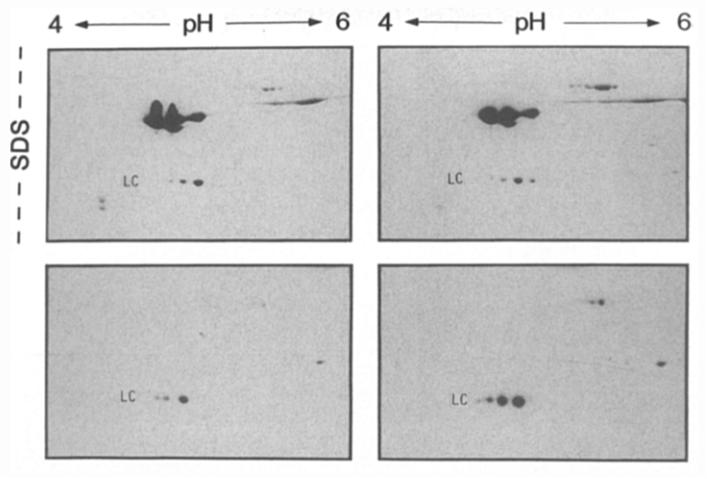

Figure 1. Two-dimensional gel electrophoretograms of arterial proteins. Top: Staining profiles. Bottom: Corresponding autoradiograms. Left: Muscle treated with PDBu. Right: Muscle treated with PDBu and then stretched.

digested and counted. These autoradiograms refer to equal amounts of protein (400 μg) applied on the gels, same exposure time and similar specific activity of [^{32}P]phosphocreatine in the muscles. Clearly, the radioactivity of LC spots in the PDBu-stretched muscle is much higher than that in the muscle treated with PDBu alone, in agreement with the data of Table 1. Major differences in the intensities of autoradiograms were also observed when untreated resting and stretched muscles were compared.

The LC isoforms separated by 2D gel electrophoresis (Figure 1) were eluted from the spots with 50 mM NH_4HCO_3 and subjected to trypsin digestion. Figure 2 compares the 2D tryptic phosphopeptide maps of LC isoforms eluted from Spots 0, 1, 2 and 3 from muscles treated with PDBu (top) or treated with PDBu and subsequently stretched (bottom). In the maps from muscles treated with PDBu, peptides E and, occasionally, F are clearly seen. However, these peptides are hardly detectable in muscles treated with PDBu and subsequently stretched, indicating no involvement of PKC in the stretch-induced phosphorylation. The peptides derived from MLCK phosphorylation are predominant in Figure 2. Peptides A and B are mainly in Spots 1 and 3, whereas peptides C and D are in Spots 0 and 2. In the phosphopeptide maps of LC from untreated rest-

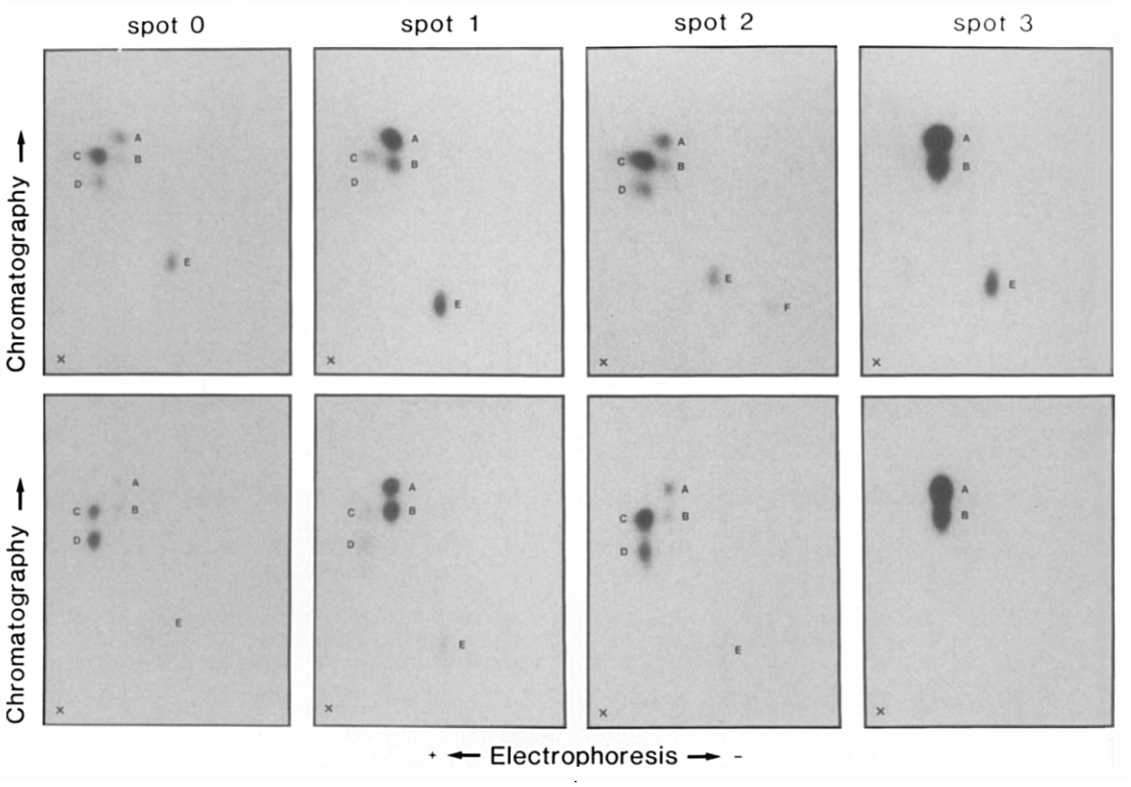

Figure 2. Autoradiograms of two-dimensional tryptic phosphopeptide maps of LC isoforms from muscles treated with PDBu (upper row) and with PDBu and then stretched (lower row). Different exposure times were used for the autoradiograms in order to compensate for the difference in [^{32}P]phosphate incorporation. A and B peptides containing serine phosphorylated by MLCK; C and D peptides containing threonine phosphorylated by MLCK; E peptide containing serine phosphorylated by PKC; F peptide containing threonine phosphorylated by PKC. The definition of Spots 0, 1, 2 and 3 is described in the text.

ing and stretched muscles, 95% of the peptides were due to MLCK; thus very little activation of PKC occurs in muscle without PDBu treatment.

Quantitation of the [^{32}P]phosphate content in the major peptide groups of LC from PDBu-treated and PDBu-stretched muscles is shown in Table 2. The data were calculated from the radioactivity of the individual peptides, from the total incorporation of [^{32}P]phosphate into LC, and the percentage distribution of radioactivity among the spots. In the PDBu-treated muscle, the total incorporation amounts to 314 millimoles [^{32}P]phosphate/mole LC; 80 millimoles of [^{32}P]phosphate are in peptides E-F demonstrating an activation of PKC by PDBu. The remaining incorporation is distrib-

Table 2. Incorporation of [^{32}P]phosphate into peptides of LC isolated from arterial muscles treated with PDBu alone or with PDBu and stretched

PDBu treated	Millimoles [^{32}P]phosphate/mol LC in tryptic peptides			
	A-B	C-D	E-F	Total
Spot 0	3	5	4	12
Spot 1	21	5	15	41
Spot 2	15	33	15	63
Spot 3	146	6	46	198
Total	185	49	80	314
PBu-treated and stretched				
Spot 0	11	41	12	64
Spot 1	98	21	10	129
Spot 2	62	136	32	230
Spot 3	472	15	10	497
Total	643	213	64	920

The powder containing the radioactive peptides, as visualized by the autoradiogram, was scraped from the cellulose sheets and the radioactivity was determined by liquid scintillation counting. The peptides are explained in the legend of Figure 2.

uted in the MLCK phosphorylated peptides: 185 millimoles in A-B and 49 millimoles in C-D. In the PDBu-stretched muscle, there are 920 millimoles total [^{32}P]phosphate incorporation, with 64 millimoles into peptides E-F; whereas peptides A-B contain 643 and C-D 213 millimoles of [^{32}P]phosphate. Accordingly, stretching the PDBu-treated muscle does not activate PKC. The stretching induces LC phosphorylation exclusively by MLCK. The same conclusion could be drawn from quantitative phosphopeptide mapping of LC from untreated resting muscle versus the stretched muscle.

DISCUSSION

The biochemical analysis of the stretch-induced LC phosphorylation in porcine carotid arteries shows clearly that it is caused by MLCK. However, as pointed out by Laher and Bevan (9), large arteries lack the Ca^{2+}-dependent intrinsic tone characteristic for the active resistance found in small arteries; thus there is a possibility that our observation holds true only for certain types of blood vessels. On the other hand, no direct

conclusion may be drawn from the inhibition of stretch-induced tone by staurosporine (9). Recently, Herbert, et al. (13) reported that the affinity of staurosporine to cAMP-dependent protein kinase and tyrosine protein kinase is greater than that to PKC, whereas the affinity to Ca^{2+}/calmodulin-dependent protein kinase roughly equals that to PKC. Therefore, staurosporine is not a specific inhibitor of PKC. Furthermore, phorbol 12-myristate 13-acetate may not be a specific activator of PKC (10) since it was shown that PDBu-induced contractile response in swine carotid medial smooth muscle may be partially mediated by MLCK (14). These considerations weaken the argument for PKC-involved LC phosphorylation in the maintenance of vascular tone but they do not rule out the involvement of other PKC-catalyzed phosphorylations.

Stretch may increase the intracellular Ca^{2+} concentration in smooth muscle (15), a requirement for MLCK activation by stretch (2). Stretch-activated Ca^{2+}-channels in the muscle membrane may participate in this process (10).

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