

### Characterization of the phosphorylatable myosin light chain in rat uterus

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The 20 kDa myosin light chain of <sup>32</sup>P-labeled rat uterus exhibited four spots on two-dimensional gel electrophoretograms; the corresponding autoradiograms revealed that three spots were radioactive. Completely dephosphorylated light chain exhibited three spots on electrophoretograms. Serine and threonine residues of the light chain were found to be phosphorylated in the uterus at a ratio of 6 to 1. During contraction, the amount of each phosphoamino acid increased proportionally to the increase in the total phosphate content of the light chain.

Since the discovery by Perry and coworkers [1] that one class of the myosin light chains can be phosphorylated, this phosphorylation has been extensively studied both in vitro and in vivo. In the case of smooth muscle, it is generally accepted that phosphorylation of the 20 kDa myosin light chain is involved in the regulation of contraction [2–4]. Early work with in vitro systems identified serine as the phosphorylation site of the smooth muscle myosin light chain [5], whereas later a threonine residue was associated with a second phosphorylation site [6–10]. In this report we characterize the phosphorylation of the 20 kDa light chain of intact rat uterus during rest and contraction.

The uteri were excised from estrogen-primed virgin rats and suspended in a muscle chamber containing physiological salt solution of a composition in mM: 118 NaCl, 5.7 KCl, 1.2 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 0.03 CaNa<sub>2</sub>EDTA, 11 glucose, 12.5 NaHCO<sub>3</sub>; the solution was bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub>, at 37°C, pH 7.4 [11]. The isometric

contractions of the isolated uterus were recorded with a transducer on a Grass 79C four-channel polygraph. For drug-induced contractions, oxytocin at 10<sup>-6</sup> M concentration was used. Spontaneous contractions were inhibited by 10<sup>-6</sup> M isoproterenol, or 10<sup>-3</sup> M EGTA. Labeling of the uterus with carrier-free [<sup>32</sup>P]orthophosphate, freezing of the uterus at different functional states, sample preparation from the frozen tissue, and two-dimensional gel electrophoresis were performed as described for artery [12]. The method for quantitation of light-chain phosphorylation is detailed in Ref. 13. The phosphoamino acids in light chain were identified according to Ref. 14.

High-resolution two-dimensional gel electrophoresis of proteins from <sup>32</sup>P-labeled rat uterus resolved the 20 kDa light chain into four spots. We numbered the spots 1 to 4 in order of increasing isoelectric points. The gel photographs (Fig. 1, first three frames of top row) with the corresponding autoradiograms (middle row) show that three of the four spots, 1–3, are radioactive, whereas spot 4 contains unphosphorylated light chain. Major changes in light-chain phosphorylation are apparent from Fig. 1 as a result of the various treatments of the uteri. The percentage distribution of the staining intensities of the 20 kDa light

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid; Ser-P, phosphoserine; Thr-P, phosphothreonine; Tyr-P, phosphotyrosine.

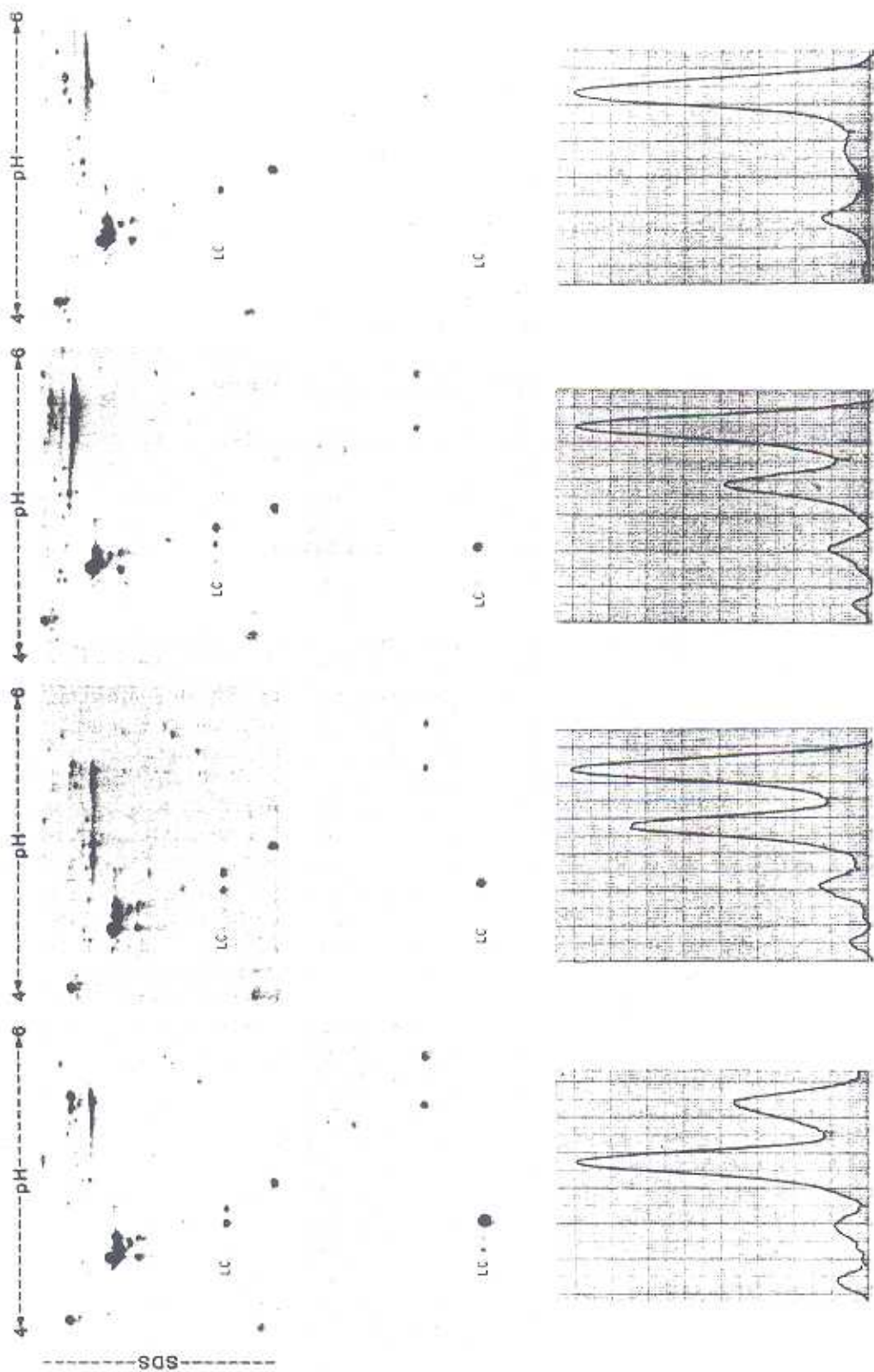


Fig. 1. Two-dimensional gel electrophoretic analysis of myosin light chain phosphorylation in uterine smooth muscle. Top row: gel staining profiles of the uterine proteins; middle row: corresponding autoradiograms; bottom row: densitometric scans of the 20 kDa light chain on the stained gels. First frame: uterine strand contracted with oxytocin for 1 min; second frame: spontaneously relaxed uterine strand; third frame: uterine strand relaxed with isoproterenol for 1 min; fourth frame: uterine strand incubated with 1 mM EGTA for 120 min. LC, 20 kDa light chain.

chain is 6, 9, 56 and 29 for the oxytocin-contracted uterine strand (first frame); 5, 11, 34 and 50 for the spontaneously relaxed uterine strand (second frame); 4, 9, 25 and 62 for the uterine strand relaxed with isoproterenol (third frame); 0, 13, 7 and 80 for the EGTA-treated uterine strand (fourth frame). While the sum of the staining intensities in the labeled spots is an indicator for changes in phosphorylation, it cannot be utilized directly for accurate quantitation. As previously shown [13], the radioactive spots 3 and 2 also contain unphosphorylated components.

From [ $^{32}$ P]phosphate content determinations [13] the following values were obtained in terms of mol phosphate/mol light chain: 0.81 for oxytocin-contracted uterus, 0.41 for spontaneously relaxed uterus, 0.18 for isoproterenol-relaxed uterus, and 0 ( $< 0.01$ ) for EGTA-treated uterus. These data illustrate the well-known fact that

uterine contraction is associated with light-chain phosphorylation [11,15]. Furthermore, they show that spontaneously relaxed uterus contains a considerable amount of phosphorylated light chain.

The case of the 120 min EGTA treatment requires special attention, because in the presence of this agent complete dephosphorylation of light chain occurred. Mathematical analysis of the [ $^{32}$ P]phosphate incorporation data in each spot with the percentage staining distribution predicted that 29% of unphosphorylated light chain is contained in spots 2 and 3 [13]. The results of EGTA treatment show that 20% of unphosphorylated light chain is present in the sum of spots 2 and 3, approximating the predicted value. The three spots remaining in the light-chain complex after EGTA treatment suggest the existence of uterine light-chain isoforms.

The following arguments suggest that the multi-

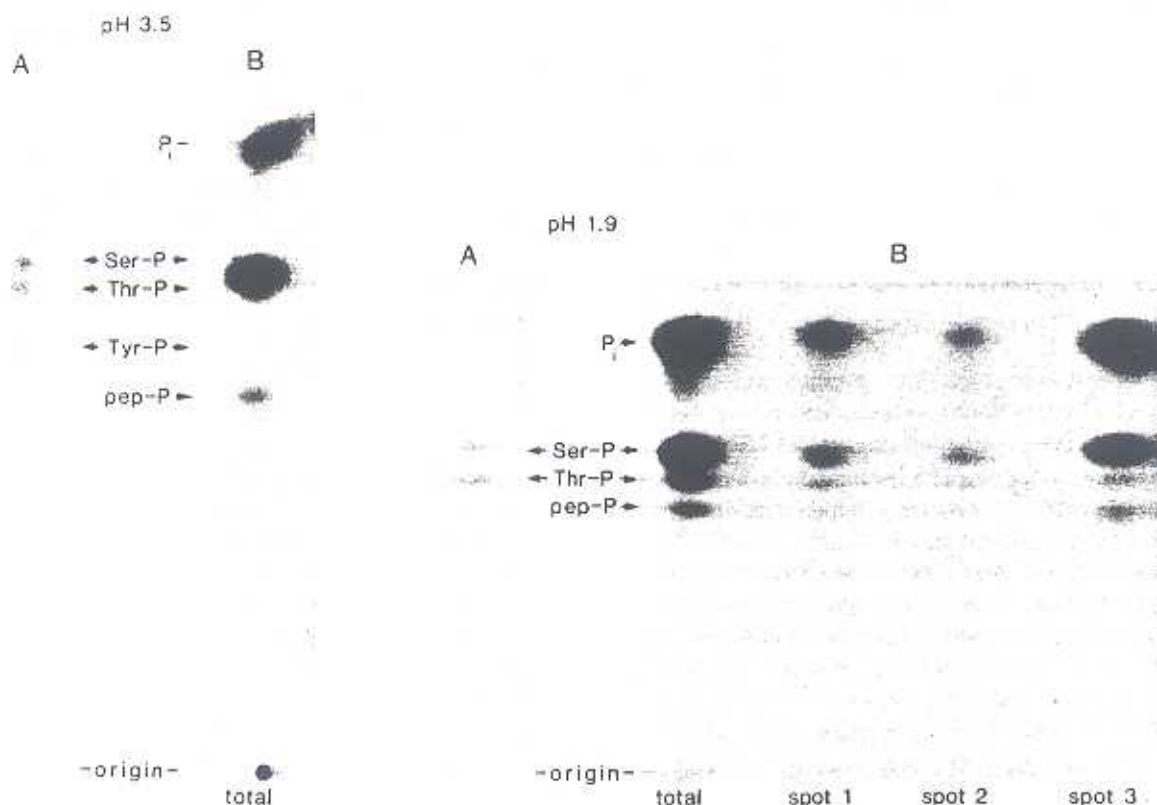


Fig. 2. Identification of phosphoamino acid residues in the myosin light chain.  $^{32}$ P-labeled uterine strands frozen in the relaxed state were analyzed at pH 3.5 and 1.9 as described [14]. A refers to the ninhydrin stain patterns of the unlabeled marker phosphoamino acids, and B refers to the autoradiograms obtained with the hydrolyzates of the light chains. At pH 1.9, in addition to the total light chain, each individual radioactive spot was analyzed. pep-P, phosphopeptide.

TABLE I  
PHOSPHOAMINO ACID CONTENT OF THE 20 kDA MYOSIN LIGHT CHAIN

For quantitation of phosphoamino acids, the light chains were extracted from SDS-polyacrylamide gel, hydrolyzed in 6 M HCl at 110°C for 0.5, 1, 2, 3 or 4 h, and electrophoresed on Kodak Chromagram 13255 cellulose sheets at pH 1.9. The spots corresponding to the phosphoamino acids were excised from the cellulose sheet and their radioactivity was measured by liquid scintillation counting. This radioactivity, expressed as percentage of the total radioactivity recovered in phosphoamino acids, inorganic phosphate and phosphopeptides, was plotted as a function of the time of hydrolysis, and the percentage of each phosphoamino acid in the light chain was calculated by extrapolating the corresponding curve to zero time. By reference to the value of mol [<sup>32</sup>P]phosphate/mol light chain, determined for each protein sample by two-dimensional gel electrophoresis, the amount of each phosphoamino acid in mol/mol light chain was calculated.

Treatment	mol [ <sup>32</sup> P]phosphate mol light chain	mol [ <sup>32</sup> P]Ser- <i>P</i> mol [ <sup>32</sup> P]Thr- <i>P</i>	mol [ <sup>32</sup> P]Ser- <i>P</i> mol light chain	mol [ <sup>32</sup> P]Thr- <i>P</i> mol light chain
Isoproterenol	0.19 ± 0.03	5.7 ± 2.6	0.16 ± 0.01	0.03 ± 0.01
Oxytocin	0.85 ± 0.17	5.8 ± 1.0	0.72 ± 0.02	0.13 ± 0.02

ple forms of uterine light chain are isoforms: (1) the four spots of arterial smooth muscle light chain [12] coelectrophorese with the four spots of uterine light chain, and by peptide mapping we have shown that the arterial light chain complex consists of isoforms [16]; (2) preelectrophoresis of gels in the presence of thioglycolate, as described in Ref. 15, had no effect on the four spots, thus they are not caused by charge modification; (3) the same two-dimensional gel electrophoretic technique which in our hands gives four uterine or arterial light-chain spots shows only two spots for the phosphorylatable light chain from skeletal muscle [17].

Another characteristic of the uterine light chain is that the phosphorylated components contain two different phosphoamino acids (Fig. 2). Electrophoresis of total light-chain hydrolyzates at pH 3.5 revealed the presence of Ser-*P* and Thr-*P*, but no Tyr-*P*. Electrophoresis at pH 1.9, which separated Ser-*P* from Thr-*P*, showed that not only the total light chain, but each radioactive light-chain spot contained both phosphoamino acids. The same results were obtained regardless of muscle treatment, with the only variable being the amount of radioactivity present in each sample.

Measurement of the radioactivity of each phosphoamino acid spot is not sufficient for quantitation of the phosphoamino acid content of <sup>32</sup>P-labeled phosphoproteins, because of the different rates of release of the phosphoamino acids from the polypeptide chain and of breakdown of

the phosphoamino acids by hydrolysis [14,18]. For this reason, we studied the time course of the hydrolysis. Table I presents the ratio of Ser-*P* to the Thr-*P* calculated for samples with low (isoproterenol-treated) and high (oxytocin-treated) levels of phosphorylation. Although there is a 4.5-fold difference in the [<sup>32</sup>P]phosphate content of the light chain, the Ser-*P*/Thr-*P* ratio remains the same, approx. 6, in the samples treated in two different ways. Accordingly, the amount of each phosphoamino acid increases proportionally as the phosphate content of light chain increases. These findings suggest that the unphosphorylated myosin light chain can be phosphorylated randomly at a serine and/or at a threonine residue.

Our findings with intact uterus are strengthened by recent studies with isolated light chain from turkey gizzard [10]; time courses of phosphorylation of the serine and threonine residues indicated a random process, although the phosphorylation rate for serine was considerably higher than that for threonine. It will be of interest to determine whether myosin light-chain kinase alone or additional kinase(s) is/are involved in the two-site phosphorylation of myosin light chain in physiologically active smooth muscle.

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