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# PROTEIN PHOSPHORYLATION DURING SPONTANEOUS CONTRACTION OF SMOOTH MUSCLE

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SUMMARY: The relationship between spontaneous contraction and protein phosphorylation of rat uterine smooth muscle was studied. Myometrial strips from estrogen-dominated rats were incubated in [$^{32}$P]orthophosphate and then frozen at various levels of isometric tension. Proteins were separated by gel electrophoresis and the incorporation of $^{32}$P was measured. Contraction was associated with the phosphorylation of one major protein (20,000 $M_r$). This phosphorylation preceded maximal tension development and dephosphorylation preceded complete spontaneous relaxation. Two-dimensional gel electrophoresis indicates that the 20,000-$M_r$ protein is the myosin light chain which has been implicated in the regulation of smooth muscle contraction.

## INTRODUCTION

The increasing evidence that phosphorylation of myofibrillar proteins may be functionally important for contraction of different muscle types has been recently reviewed (1). In the case of smooth muscle, there is evidence both for (2-9) and against (10,11) the hypothesis that $Ca^{2+}$-dependent phosphorylation of the 20,000-$M_r$ myosin light chain is required for actin-activation of myosin ATPase activity. These contradictory findings are based on studies using isolated actomyosin preparations. An alternative approach is to measure the phosphorylation or dephosphorylation of specific proteins in intact smooth muscle under conditions of contraction or relaxation. In the present investigation, we determined the relationship between protein phosphorylation and spontaneous contraction of rat myometrium. Contraction of uterine smooth muscle was associated with the reversible phosphorylation of a 20,000-$M_r$ protein. The results support the hypothesis that contraction and relaxation of smooth

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muscle is regulated, at least in part, by phosphorylation and dephosphorylation of the myosin light chain.

## MATERIALS AND METHODS

Labelling of tissues. Virgin Holtzman rats (175-225 g) were injected subcutaneously with 50 ug of estradiol benzoate (dissolved in peanut oil) 48 hours before sacrifice. Rats were killed by cervical dislocation and two myometrial strips were prepared from each animal as previously described (12). The incubation solution was (in mM): NaCl, 125; KCl, 2.4; MgCl, 0.5; $CaCl_2$, 1.8; glucose, 11.0; and Tris-HCl[1], 23.8 (pH 7.0). The solution was aerated with 100% $O_2$ and maintained at 37°C. Pairs of muscle strips were incubated for 30 min. in the above solution and then incubated in fresh solution to which was added 200 uCi/ml carrier-free [$^{32}$P]ortho-phosphate. Tissues were then suspended in isolated organ baths for recording isometric tension (12) and were washed 5 times with the phosphate-free incubation solution. Resting tension was gradually increased over 15 to 20 minutes until maximal spontaneous contractions were obtained. Paired myometrial strips at various levels of isometric tension were frozen with a modified Wollenberger clamp cooled in isopentane at -80°C.

Preparation of samples. For one-dimensional electrophoresis, frozen muscle samples were powdered at -80°C and mixed with 1 ml of ice-cold 10% trichloroacetic acid. After 15 min at 0°C, the mixture was centrifuged at 3000 x g for 10 min and the acid was extracted with diethyl ether. The precipitated proteins were solubilized by boiling for 2 min in 500 ul of 6% SDS and 10 mM Tris-HCl (pH 7.0). Before electrophoresis, proteins were boiled for an additional 3 min. in a solution containing a final concentration of 1.0 mg/ml protein, 5% SDS, 2% 2-mercaptoethanol, 1 mM EDTA, 8% sucrose, 8 mM Tris-HCl, pH 7.0 and 0.1% bromophenol blue.

For two-dimensional electrophoresis frozen muscle samples were ground and then homogenized with a Polytron at half-maximal speed for 30 s in a solution containing: 100 mM NaF, 80 mM sucrose, 10 mM EDTA and 10 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4 (14). The homogenate was centrifuged at 100,000 x g for 1 hour, the pellet boiled for 3 minutes in 1% SDS, 10 mM Tris-HCl, pH 7.0. Protein samples (80 ug) were loaded onto isoelectric focusing gels in a solution containing 1% SDS, 5% 2-mercaptoethanol, 0.2% Ampholines (pH 5-7) and 10 mM Tris-HCl (pH 7.0).

Electrophoresis. One-dimensional SDS electrophoresis was carried out on 7-18% linear polyacrylamide gradients (13). Two-dimensional gel electrophoresis, gel drying, staining and destaining were carried out as described by O'Farrell (15) with the modifications of Storti et al. (16). Molecular weight standards were run on all slab gels. Chicken gizzard actomyosin was kindly supplied by Dr. Kate Bárány, University of Illinois Medical Center.

Determination of $^{32}$P incorporation into protein, nature of the phosphate bond, and protein. Incorporation of radioactivity was estimated after one-dimensional SDS-polyacrylamide gel electrophoresis by autoradiography and microdensitometry, and by slicing the gel for liquid scintillation

[1] Abbreviations are: tris(hydroxymethyl)aminomethane, Tris; sodium dodecyl sulfate, SDS.

counting (13, 14). Similar results were obtained with these two methods. Treatment of the phosphorylated material with pronase, ribonuclease, chloroform-methanol (3:1), and hot NaOH solution (14) indicated the isotopic phosphorus in the 20,000-$M_r$ material was bound to protein via an ester linkage. Measurement of protein was by the Hartree modification (17) of the Lowry method.

## RESULTS AND DISCUSSION

The spontaneous contraction of rat myometrium was associated with the phosphorylation of a 20,000-$M_r$ protein (Fig. 1). Incorporation of $^{32}P$ into this protein was approximately doubled in myometria frozen in the maximally contracted state as compared to those frozen in the relaxed state (Table 1). If the 20,000-$M_r$ protein is involved in the regulation of the spontaneous contractions, then its increased phosphorylation should precede the development of maximal spontaneous contraction, and its dephosphorylation should precede maximal relaxation. These, in fact, were the results obtained (Table 1, Fig. 2). The identity of this protein was not definitively established, but two-dimensional gel electrophoretic results (Fig. 3) are consistent with the idea that the protein is the myosin light chain, since the apparent isoelectric point of the

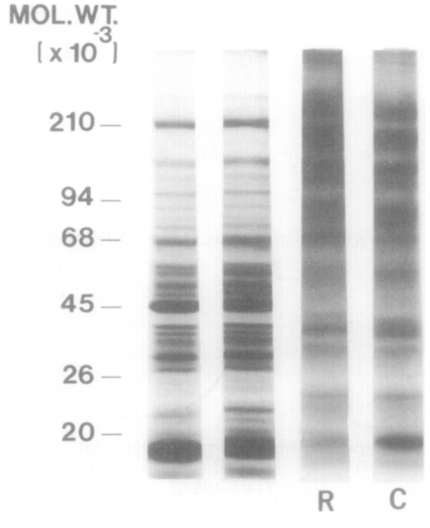


Fig. 1. Protein staining pattern after SDS-polyacrylamide gel electrophoresis (left pair) and autoradiograms of protein (right pair) from resting (R) and spontaneously contracted (C) rat myometria.

Table 1. Phosphorylation of 20,000-$M_r$ protein during spontaneous contraction and relaxation of rat myometrium.

| Percent Maximal Tension | Percent of Control $^{32}P$ Incorporation | Number of Experiments |
|---|---|---|
| 59 ± 3.2 (before maximal contraction) | 213 ± 16 | 9 |
| 98 ± 2.0 | 227 ± 13 | 10 |
| 45 ± 5.5 (after maximal contraction)[2] | 106 ± 28 | 4 |

[1] Data are expressed as a percent of the $^{32}P$ incorporation into the paired control (resting) myometrial strip obtained from the same rat(mean ± S.E.). The value for the control strip was taken as 100 percent. Data were obtained by microdensitometry using peak height (13, 14) to estimate $^{32}P$ incorporation.

[2] Myometrial strips were frozen during the relaxation phase following peak isometric tension.

phosphoprotein (5.0) was the same as that reported for the myosin light chain from bovine aorta (4). In addition, this protein was found to co-migrate with the 20,000-$M_r$ myosin light chain of chicken gizzard actomyosin in several gel systems, including 12% SDS-polyacrylamide gels and 10.3% polyacrylamide-urea gels (18), (results not shown).

The increased phosphorylation of the 20,000-$M_r$ protein does not appear to be mediated by cyclic nucleotides. The rat myometrium, selected for these studies, does not exhibit increased cyclic GMP levels during spontaneous contraction (12). In contrast, KCl and drug-induced

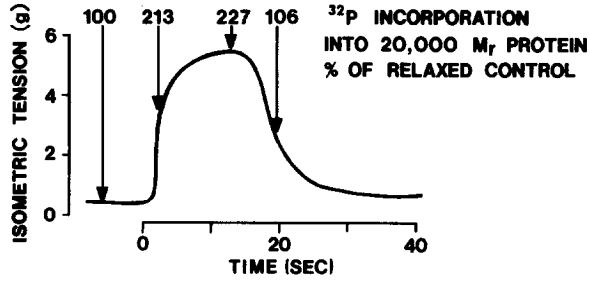


Fig. 2. Mechanical response of spontaneously contracting rat myometrium. Contractions occurred about one per minute. Also shown are the means from Table 1 for isotopic phosphorous incorporation at certain levels of isometric tension.

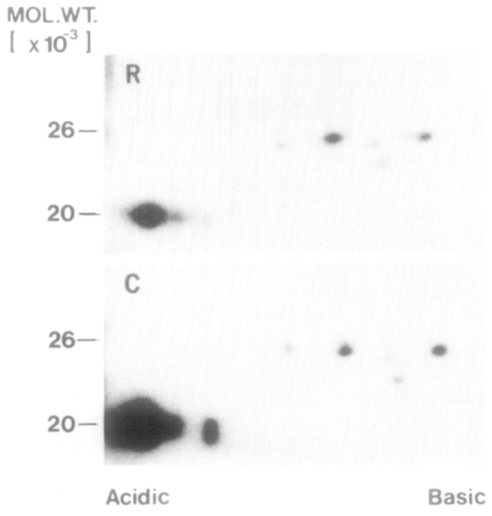


Fig. 3. Autoradiograms of $^{32}$P-labelled proteins separated by two-dimensional gel electrophoresis. Proteins are from resting (R) and spontaneously contracted (C) rat myometrium. The results shown are representative of 4 experiments.

contraction of many other types of smooth muscles is associated with a large increase in cyclic GMP levels (19, 20). Therefore, the previously reported associations between phosphorylation and drug-induced contraction (18, 21) might be due to an increased cellular concentration of this cyclic nucleotide, rather than elevation of intracellular calcium activity. This is probably not the case for the present results, nor for sarcolemma-free chicken gizzard bundles which exhibit increased phosphorylation of the myosin light chain when exposed to $Ca^{2+}$ (22). Thus, the increased phosphorylation reported here is most likely due to an elevation of intracellular $Ca^{2+}$ activity, as expected from results obtained with isolated actomyosin from several types of smooth muscle (2-3). In summary, our results suggest that spontaneous contraction of rat uterine smooth muscle is associated with increased myosin light chain phosphorylation; the temporal relationship with contraction support the hypothesis that this phosphorylation is physiologically significant.

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