Communication

Dephosphorylation of Myosin by the Catalytic Subunit of a Type-2 Phosphatase Produces Relaxation of Chemically Skinned Uterine Smooth Muscle*

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It is now well-established that phosphorylation of the 20,000-dalton light chain of smooth muscle myosin (LC20) is a prerequisite for muscle contraction. However, the relationship between myosin dephosphorylation and muscle relaxation remains controversial. In the present study, we utilized a highly purified catalytic subunit of a type-2, skeletal muscle phosphoprotein phosphatase (protein phosphatase 2A) and a glycinated smooth muscle preparation to determine if myosin dephosphorylation, in the presence of saturating calcium and calmodulin, would cause relaxation of contracted uterine smooth muscle. Addition of the phosphatase catalytic subunit (0.28 μM) to the muscle bath produced complete relaxation of the muscle. The phosphatase-induced relaxation could be reversed by adding to the muscle bath either purified, thiophosphorylated, chicken gizzard 20,000-dalton myosin light chains or purified, chicken gizzard myosin light chain kinase. Incubation of skinned muscles with adenosine 5′-O-(thiotriphosphate) prior to the addition of phosphatase resulted in the incorporation of 0.93 mol of PO₄/mol of LC20 and prevented phosphatase-induced relaxation. Under all of the above conditions, changes in steady-state isometric force were associated with parallel changes in myosin light chain phosphorylation over a range of phosphorylation extending from 0.01 to 0.97 mol of PO₄/mol of LC20. We found no evidence that dephosphorylation of contracted uterine smooth muscles, in the presence of calcium and calmodulin, could produce a latch-state where isometric force was maintained in the absence of myosin light chain phosphorylation. These results show that phosphorylation or dephosphorylation of the 20,000-dalton myosin light chain is adequate for the regulation of contraction or relaxation, respectively, in glycinated uterine smooth muscle.

Previous experiments using chemically skinned smooth muscle preparations have provided evidence that phosphorylation of the 20,000-dalton myosin light chain (LC20) is both necessary and sufficient for isometric force development and maintenance (1–4). Using ATPγS¹ to produce nearly irreversible thiophosphorylation of the 20,000-dalton light chain subunits of myosin in chemically skinned smooth muscle, Kerrick and colleagues (1) demonstrated that isometric force could be maintained in the absence of calcium. More recent studies have shown that the addition of a calcium-independent myosin light chain kinase to a skinned muscle preparation could stimulate isometric force development and associated myosin light chain phosphorylation (5). Others, using both skinned and intact smooth muscle preparations, have demonstrated that isometric force development was associated with increased myosin phosphorylation while isometric force maintenance, on the other hand, was not always accompanied by elevated myosin phosphorylation (6–8). It has been suggested by Murphy and colleagues that relaxation of vascular and tracheal smooth muscle may require both dephosphorylation of the myosin light chain and a reduction of the intracellular calcium concentration (6). These same investigators have more recently demonstrated calcium dependent force maintenance in the absence of elevated myosin phosphorylation in skinned vascular smooth muscle (7). We have previously reported that, for intact uterine smooth muscle stimulated by a number of mechanistically different agonists, changes in steady-state myosin phosphorylation were associated with proportional changes in isometric force (23, 24, 29). We never observed isometric force maintenance in the absence of a significant elevation of myosin phosphorylation from control levels. This suggests that phosphorylation independent force maintenance may not occur in uterine smooth muscle. The studies reported here were undertaken to establish whether myosin light chain dephosphorylation, in the presence of calcium, is sufficient to induce muscular relaxation in skinned uterine smooth muscle.

EXPERIMENTAL PROCEDURES

Protein Purification—A low molecular weight phosphoprotein phosphatase catalytic subunit was purified from rabbit skeletal muscle by a modification of the procedures of Silberman et al. (9) and Tung et al. (10). The enzyme preparation had a specific activity of 10,000 units/mg using [32P]phosphorylase at 2 mg/ml as substrate, and was not inhibited by the heat stable phosphatase inhibitor-2. The 20,000-dalton myosin light chain was purified from chicken gizzard smooth muscle as previously described (11). Myosin light chain kinase was purified from chicken gizzard as described elsewhere (12). Calmodulin was purified from bovine testis using the method of Klee et al. (13).

Chemical Methods—Purified chicken gizzard 20,000-dalton myosin light chains were phosphorylated by established procedures (14). Protein concentrations were determined by the method of Lowry et al. (15).

Gel Electrophoresis—Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was carried out by a modification (16) of the Laemmli procedure (17). A gradient of 6–20% acrylamide was used to make the gels. Polypeptide bands were visualized by the silver stain method (18).

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¹ The abbreviations used are: ATPγS, adenosine 5′-O-(thiotriphosphate); MOPS, 4-morpholino propane sulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PCS, phosphatase catalytic subunit; CS, contracting solution.
Muscle Preparation—Glycercinated uterine smooth muscles were prepared from estrogen primed Sprague-Dawley rats as described elsewhere (19) and stored frozen at -70 °C. At the time of the experiment, the uterine segments were thawed and the superficial layer of longitudinal muscle was dissected free from the underlying circular muscle and endometrium. The longitudinal muscle layer was approximately 50 μm thick and contained well preserved contractile filaments as previously shown by electron microscopy (19). Segments of longitudinal muscle (5 x 3 x 0.05 mm) were attached, using a cyanoacrylate adhesive, to an electromagnetic muscle ergometer (Cambria Technology, Model 903) system. The muscle bath consisted of a small polypropylene cup (200 μl) which was rotated about the muscle (approximately 4 Hz) to provide stirring.

Solutions—All purified proteins were in Buffer A which contained: 20 mM MOPS (pH 7.0), 100 mM potassium glutonate, 1 mM diithiothreitol, 2 mg/ml bovine serum albumin, and 10% glycerol. The skinned muscles were contracted in contracting solution (CS) which contained equal volumes of Buffer B and Buffer A alone, or Buffer A containing purified protein. Buffer B contained: 80 mM, imidazole, 464 μM calcium carbonate, 100 mM potassium glutonate, 13.8 mM Na2ATP, 14 mM magnesium acetate, 20 μM calmodulin, and 2 mM diethiothreitol, at pH 7.0. This resulted in a CS with 292 μM calcium carbonate, 6.8 mM Na2ATP, 7 mM magnesium acetate, 100 mM potassium glutonate, 40 mM imidazole, 10 μM calmodulin, and 1 mM diethiothreitol. This concentration of total calcium was empirically determined to produce a maximal contraction in the presence of 10 μM calmodulin. Comparison of the contractile response of skinned muscles contracted with an EGTA-buffered solution without Buffer A indicated that the apparent free calcium concentration of the CS was approximately 30 μM. The relaxing solution contained: 40 mM imidazole, 100 mM potassium glutonate, 6.8 mM Na2ATP, 7 mM magnesium acetate, 1 mM diethiothreitol, and no added calcium or calmodulin at pH 7.0. Metal ion chelators (EDTA and EGTA) were avoided because of the established sensitivity of many phosphoprotein phosphatases to inactivation by chelation of tightly bound trace metal ions (20–22).

Myosin Light Chain Phosphorylation—Muscles were frozen for myosin light chain phosphorylation measurement by immersion in dry ice-cooled acetone. The stoichiometry of 20,000-dalton myosin phosphorylation and isometric force (in the presence of saturating calcium and calmodulin) was examined over a range of myosin phosphorylation and isometric force was apparently independent of the muscle bath, restored both isometric force and myosin phosphorylation as shown by the second contraction and associated gels in Fig. 1A. Boiling the phosphatase containing buffer for 30 min completely eliminated phosphatase activity in vitro as well as the effects on skinned muscle contraction and phosphorylation. To demonstrate that the phosphatase-induced relaxation and dephosphorylation were causally related, three experimental methods were employed to either inhibit or reverse dephosphorylation by the phosphatase catalytic subunit. First, glycercinated muscles were pretreated with ATPγS prior to exposure to the phosphatase. As shown in Fig. 1B, addition of ATPγS to the muscle bath resulted in a 2-fold increase in isometric force which corresponded to an increase in the stoichiometry of myosin phosphorylation from 0.45 mol of PO4/mol of LC20 to 0.93 mol of PO4/mol of LC20. The increased force and phosphorylation were not diminished by the subsequent removal of ATPγS and were only slightly reduced by the addition of phosphatase. A second method of phosphatase inhibition, shown in Fig. 1C, utilized purified, thiophosphorylated, chicken gizzard 20,000-dalton myosin light chains. The first contraction in Fig. 1C shows the effect of adding thiophosphorylated light chains to a skinned muscle which had been contracted in the presence of saturating calcium and calmodulin. Isometric force increased by approximately 2-fold, presumably due to substrate inhibition of endogenous myosin light chain phosphatase activity. The addition of phosphatase catalytic subunit produced complete relaxation which was subsequently reversed, in a concentration-dependent fashion, by the addition of thiophosphorylated myosin light chains. The removal of both the catalytic subunit and the thiophosphorylated light chains resulted in full contraction of the muscle. Finally, as shown in Fig. 1D, the addition of purified, chicken gizzard myosin light chain kinase reversed the phosphatase-induced relaxation and myosin dephosphorylation. By using the experimental approaches described above to alter the level of isometric force and myosin phosphorylation (see Fig. 1A, A–D), the relationship between myosin phosphorylation and isometric force (in the presence of saturating calcium and calmodulin) was examined over a range of myosin phosphorylation from 0.01 to 0.97 mol of PO4/mol of LC20. Some muscles were contracted from a relaxed state, using either calcium or calmodulin, calcium-calmodulin plus ATPγS, or calcium-calmodulin plus myosin light chain kinase, and frozen for phosphorylation determination. Others where first contracted with calcium-calmodulin and then either partially or completely relaxed by the addition of phosphatase catalytic subunit. Partial relaxation to intermediate, steady-state levels of isometric force was achieved by using phosphatase concentrations ranging from 0.07 to 0.28 μM. And finally, muscles were contracted with calcium-calmodulin, relaxed with phosphatase catalytic subunit, and recontracted by removing the catalytic subunit or by adding myosin light chain kinase. The results of these experiments are summarized in Fig. 2. There was a significant correlation (correlation coefficient of 0.96, p < 0.001) between the stoichiometry of myosin phosphorylation and the steady-state isometric force. Moreover, the correlation between myosin phosphorylation and isometric force was apparently independent of the method and direction by which a particular level of force and myosin phosphorylation was achieved.
DISCUSSION

The results of this study demonstrate that a highly purified catalytic subunit of a type-2, phosphoprotein phosphatase can dephosphorylate the myosin 20,000-dalton light chains in a glycerinated uterine smooth muscle preparation. Moreover, our results show that dephosphorylation, in the presence of saturating calcium and calmodulin, produces complete relaxation. Previous studies with chemically skinned smooth muscle have shown that myosin phosphorylation catalyzed by a calcium- and calmodulin-independent myosin light chain kinase, in the presence of EGTA, produces contraction (5). Taken together, these results indicate that phosphorylation and dephosphorylation of the 20,000-dalton light chain of myosin are sufficient to produce contraction and relaxation, respectively, of skinned smooth muscle.

Force maintenance in the face of myosin light chain dephosphorylation was not observed in the present study despite the presence of saturating concentrations of calcium and calmodulin. This finding suggests that a "latch-state," as originally proposed by Murphy and his colleagues (6, 7, 27), may not be present in uterine smooth muscle. According to their hypothesis, dephosphorylation of actomyosin in the presence of calcium results in the formation of calcium-dependent latch-bridges which are able to maintain previously developed isometric force. This hypothesis was proposed, in
part, to explain calcium-dependent isometric force maintenance in vascular and nonvascular smooth muscles (30). Certain nonvascular smooth muscle types might account for the regulation of isometric force over a wide range of phosphorylation levels (i.e. 0.01-0.97 mol of PO4/mol of LC3P). Moreover, this relationship was similar for both contraction and relaxation. Using a purified catalytic subunit of a type-2 phosphatase to dephosphorylate myosin in the presence of saturating calcium and calmodulin, we were unable to demonstrate the presence of a second regulatory system that provides for force maintenance in the absence of myosin phosphorylation.

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Fig. 2. Relationship between myosin light chain phosphorylation and steady-state isometric force maintenance for skinned uterine smooth muscle. Skinned muscles were contracted as shown in Fig. 1. CS (contracting solution containing saturating calcium and calmodulin) + 4 mM ATPyS followed by CS + 0.28 μM PCS (see Fig. 1B); CS + 4 mM ATPyS followed by CS + 0.28 μM PCS (see Fig. 1A); CS only (see Fig. 1A); CS + 0.67–0.28 μM PCS (see Fig. 1A); relaxing solution only (see "Experimental Procedures"); A, recontraction with CS following complete relaxation by 0.28 μM PCS (see Fig. 1A); A, CS + 12 μM myosin light chain kinase; A, CS + 0.28 μM PCS + 12 μM myosin light chain kinase (see Fig. 1D).

3 J. R. Haeberle, D. R. Hathaway, and A. A. DePaoli-Roach, unpublished experiments.