Effect of Multiple Phosphorylations of Smooth Muscle and Cytoplasmic Myosins on Movement in an \textit{in Vitro} Motility Assay*

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The \textit{Nitella}-based \textit{in vitro} motility assay developed by Sheetz and Spudich (Sheetz, M. P., and Spudich, J. A. (1983) Nature 303, 51-35) is a quantitative assay for measuring the velocity of myosin-coated beads over an organized substratum of actin. We have used this assay to analyze the effect of phosphorylation of various sites on the 20,000-Da light chain of smooth muscle and cytoplasmic myosins. Phosphorylation by myosin light chain kinase at serine 19 on the 20,000-Da light chain subunit of smooth muscle myosin from turkey gizzard, bovine trachea and aorta, and of cytoplasmic myosin from human platelets was required for bead movement. The individual phosphorylated myosin-coated beads moved at characteristic rates under the same conditions (turkey gizzard myosin, 0.2 \mu m/s; aorta or trachea myosin, 0.12 \mu m/s; and platelet myosin, 0.04 \mu m/s; in contrast, rabbit skeletal muscle myosin, 2 \mu m/s). Myosin light chain kinase can also phosphorylate threonine 18 in addition to serine 19, and this phosphorylation resulted in an increase in the actin-activated MgATPase activity of HMM over that obtained by phosphorylation of serine 19 alone (6-8). Smooth muscle myosin is also a substrate for protein kinase C, the Ca\textsuperscript{2+}/phospholipid-dependent enzyme. Protein kinase C has been shown to phosphorylate two to three sites in the first nine amino acids of the 20,000-Da light chain subunit of smooth muscle myosin (9-11). These are threonine 9, which is the site preferred by protein kinase C, and either serine 1 or serine 2. Phosphorylation of myosin by protein kinase C alone has little or no effect on the actin-activated MgATPase activity, whereas phosphorylation of smooth muscle myosin by protein kinase C and MLC kinase reduces the actin-activated MgATPase activity of myosin through an effect on the $K_{\text{act}}$ for actin (9, 11).

Thus, in addition to the primary regulation of smooth muscle myosin's interaction with actin by MLC kinase phosphorylation of serine 19, there appear to be several phosphorylations of myosin which may be best described as "modulatory." When bound to actin, tropomyosin also has a modulatory role on the actin-activated MgATPase activity of smooth muscle myosin (12). The actin-activated MgATPase activity of smooth muscle myosin is usually 2-4-fold higher when assayed with tropomyosin-actin than when assayed with actin alone. The ability of actin to activate the MgATPase activity of myosin has been a powerful tool in studying the \textit{in vitro} regulation of myosin-actin interaction. Recently, other assays of myosin's function have been developed which allow the relative sliding velocity of myosin and actin to be measured \textit{in vitro} (13-17). One of these assays involves the addition of myosin-coated polymer beads to oriented actin cables which are exposed by dissection of \textit{Nitella axillaris}, a fresh water alga (14). This assay has been shown to give quantitative data on the velocity of several myosin-coated beads over these actin cables and seems to correlate with the unloaded shortening velocity (or $V_{\max}$) of muscle contraction (13, 18-21).

In this report we describe the effects of phosphorylation of amino acids in addition to that of serine 19 on the movement of smooth muscle cytoplasmic myosin-coated beads in the \textit{Nitella}-based \textit{in vitro} motility assay and compare the results of this assay with those of the more conventional steady-state kinetic analysis of the actin-activated MgATPase activity. In addition, we examine the effect of troponyosin on the movement of myosin-coated beads and on the kinetics of...
actin-myosin interaction. Some of this material has been presented in preliminary form (22).

MATERIALS AND METHODS

Preparation of Proteins—All procedures were performed at 4°C. Myosin was prepared from fresh turkey gizzards by a modification of the method of Sobieszek and Bremel (23). Fresh turkey gizzard muscle (typically 500 g) was ground in a meat grinder and was homogenized in a Waring blender for 30 in 4 volumes of Buffer A (50 mM NaCl, 10 mM MOPS (pH 7.0), 1 mM MgCl₂, 1 mM EGTA, 3 mM NaN₃, 0.1 mM dithiothreitol, 0.1 mM PMSF) and stirred with an overhead mixer at 4°C for 45 min before sedimentation at 13,000 × g for 20 min, and the supernatant was discarded. The pellet was manually resuspended in Buffer A plus 0.1 mM PMSF (without the Triton X-100) and then resedimented. This washing with Buffer A plus 0.1 mM PMSF was continued for two additional times. The washed "myofibrils" were resuspended with the aid of a brief (5-s) homogenization in the Waring blender in extraction buffer (40 mM NaCl, 40 mM MOPS (pH 7.2), 4 mM EGTA, 2 mM EDTA, 10 mM ATP, 1 mM dithiothreitol, 0.1 mM PMSF) and stirred with an overhead mixer at 4°C for 45 min before sedimentation at 13,000 × g for 20 min. The supernatant containing crude actomyosin was filtered through cheesecloth and was made 0.5 M in NaCl, 20 mM in magnesium sulfate, and an additional 5 mM in ATP by the addition of stock solutions of 0.5, 0.1, and 1 M, respectively. Saturated ammonium sulfate was slowly added with vigorous stirring to 40% saturation. The sample was sedimented at 13,000 × g for 10 min, and the supernatant was filtered through cheesecloth. It was then brought to 55% saturation by the addition of saturated ammonium sulfate, sedimented at 13,000 × g for 20 min, and the pelleted solution was dialyzed at 25°C for 5 min against 100 mM in NaCl, 50 mM in MgCl₂, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM diithiothreitol, and dialyzed against 20 liters of the same solution overnight. The following day the precipitated solution was slowly diluted with 1 volume of cold H₂O and collected by sedimentation at 13,000 × g for 20 min. The supernatant could be used to prepare tropomyosin (see below) or was resuspended in 0.1 M NaCl, 10 mM MgCl₂, 10 mM MOPS (pH 7.0), 1 mM diithiothreitol, and resedimented again at 13,000 × g for 20 min. The crude myosin was resuspended with the aid of a Teflon-glass homogenizer in Buffer B at a concentration of 20 mg/ml. The yield from 500 g of ground gizzard muscle was typically 2 g of myosin. To prepare myosin for use in the motility assays, 100 mg of crude myosin was chromatographed on a Sepharose 4B column (5 × 90 cm) which was equilibrated and developed in 0.5 M NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM dithiothreitol, 3 mM NaN₃. Just prior to applying the myosin to the column, the sample was made 0.5 M in NaCl, 10 mM in MgCl₂, and 30 mM in ATP by the addition of stock solutions of 0.5, 0.1, and 1 M, respectively. If the actin contamination of the crude myosin was more than about 5% (as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis), 100 ml of 5 mM MgCl₂ and 5 mM ATP in the column buffer was sometimes applied in order to facilitate the separation of actin from the myosin. The myosin-containing fractions were pooled and concentrated against 25% (v/v) (Ambínt 5R) and resedimented at 13,000 × g for 20 min, and then the supernatant was applied to a Sephacryl S-300 column. The peak containing pure HMM was collected, and the milk was dialyzed against 100 mM in NaCl, 50 mM in MgCl₂, 5, 1, 0.5, 0.1, and 0.05 M in NaCl, and then dialyzed against 0.5 M in NaCl by the addition of stock solutions of 0.5, 0.1, and 1 M, respectively. Saturated ammonium sulfate was slowly added with vigorous stirring to 40% saturation. The sample was sedimented at 13,000 × g for 20 min. The supernatant was filtered through cheesecloth, brought to 55% saturation by the slow addition of saturated ammonium sulfate, and the precipitate was collected by sedimentation at 13,000 × g for 20 min. The pellet was dissolved in 25 mM NaCl, 10 mM MgCl₂, 10 mM MOPS (pH 7.0), 1 mM dithiothreitol, 3 mM NaN₃, 0.1 mM PMSF, 5 mg/ml leupeptin and dialyzed against the same solution overnight. The following day the precipitated myosin was collected by sedimentation at 13,000 × g for 20 min and resuspended in the above buffer. If the above dialyzed sample contained large amounts of an soluble sulfonate derivative of myosin, the sample was dialyzed against 25°C for 10 min, and 100 mg of crude myosin was dialyzed at 25°C and then dialyzed against 100 mM in NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.1 mM EGTA, 0.2 mM CaCl₂, 5 × 10⁻⁷ M calmodulin, 2 mM ATP, 0.5 mM dithiothreitol, 1 mg/ml myosin, and 1.2 μg/ml MLCK kinase at 25°C for 40 min. Smooth muscle myosin and HMM were dialyzed against serine 19 on the 20,000 Da light chain subunit (1 mol of P₅/μmol of light chain) using the following conditions: 20 mM NaCl, 10 mM MOPS (pH 7.4), 8 mM MgCl₂, 0.1 mM EGTA, 0.2 mM CaCl₂, 5 × 10⁻⁷ M calmodulin, 2 mM ATP, 0.5 mM dithiothreitol, 1 mg/ml myosin, and 1.2 μg/ml MLCK kinase at 25°C for 40 min. Smooth muscle myosin and HMM were phospho- rylated at serine 19 on the 20,000 Da light chain subunit (1 mol of P₅/μmol of light chain) using the following conditions: 20 mM NaCl, 10 mM MOPS (pH 7.4), 8 mM MgCl₂, 0.1 mM EGTA, 0.2 mM CaCl₂, 5 × 10⁻⁷ M calmodulin, 2 mM ATP, 0.5 mM dithiothreitol, 1 mg/ml myosin, and 1.2 μg/ml MLCK kinase at 25°C for 40 min. The extent of light chain phosphorylation was monitored by autoradiography of 12.5% sodium dodecyl sulfate-polyacrylamide gels. The sites of phosphorylation of myosin light chains were determined by two-dimensional tryptic peptide mapping (9) of the phosphorylated light chain following sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (31).

Assays—The actin-activated MgATPase activity of turkey gizzard HMM was carried out as described by Sellers et al. (32) using the method of Pollard and Korn (33).

The Nitella-based in vitro motility assay was performed essentially as described by Sheetz et al. (14). N. azillaris were routinely dissected in a buffer containing 10 mM KC1, 10 mM imidazole (pH 7.2), 5 mM MgCl₂, 1 mM ATP, 1 mM EGTA, 2 mM dithiothreitol, 0.01 μM phalloidin, 10 mM sucrose at 35 °C. Myosin was diluted to 100 μg/ml in 50 μl of this buffer in the presence of 5 μl of 6-μm polystyrene beads (Polysciences, Inc.). After a few seconds of incubation, a portion of this mixture was mixed with an equal portion of 0.5 mM sucrose and placed in a sonicating water bath for 1-2 s. A 1-2 μl aliquot of beads was added with a pipette tip directly above the dissected Nitella, and the beads were allowed to settle and attach for about 2 min before initiating the videotaping. The bead movement was visualized using a Zeiss inverted microscope with an Hitachi color camera interfaced with a Panasonic AG6300 video recorder. Usually a given field was videotaped for 5 min and would contain between 2 and 20 beads. For any given experiment several fields would be recorded on two independently dissected segments of Nitella. The velocity of the beads was quantitated using the computer-assisted method described by Sheetz et al. (14). Typically the velocities of 10-30 beads were measured, and the mean of the mean smoothed velocity was taken to obtain the reported value. Student’s t test was used for statistics.

RESULTS

We measured the velocity of beads coated with phosphorylated smooth muscle myosin from turkey gizzard, bovine aorta, and bovine trachea and with phosphorylated cytoplasmic myosin from human platelets in the Nitella-based in vitro motility assay under our standard conditions (10 mM KC1, 10 mM imidazole (pH 7.2), 5 mM MgCl₂, 1 mM ATP, 1 mM EGTA, 10 mM sucrose, 2 mM dithiothreitol, 0.01 μM phalloidin at 25 °C). However, each of these phosphorylated myosin-coated beads moved at their own characteristic velocity (Table I). Phosphorylated myosin-coated beads from turkey gizzard moved at about 0.2 μm/s, whereas smooth muscle myosin-coated beads from the two bovine tissues moved somewhat slower (about 0.1-0.13 μm/s). Myosin-coated beads from human platelets moved considerably slower (at about 0.04 μm/s). This slow velocity of phosphorylated human platelet myosin-coated beads appeared to be characteristic for vertebrate cytoplasmic myosins since phosphorylated myosin-coated beads from bovine thymus and from turkey intestinal epithelial brush border also moved at this slow rate (data not shown). In contrast, rabbit skeletal muscle myosin-coated beads moved at about 1.9 μm/s under these conditions.

The movement of all of these vertebrate smooth muscle or cytoplasmic myosin-coated beads was totally dependent on the phosphorylation of the 20,000-Da light chain by MLC kinase under these assay conditions. This was directly demonstrated by the use of a calcium-insensitive proteolytic fragment of MLC kinase. If unphosphorylated smooth muscle or cytoplasmic myosin-coated beads were added onto the Nitella, no movement of the beads was observed. Subsequent addition of the calcium-insensitive MLC kinase to the same solution resulted in the movement of all of the beads at the same velocity as the control MLC kinase phosphorylated myosin.

In the above studies, the myosins were phosphorylated at serine 19, which is the preferred phosphorylation site for MLC kinase. If the MLC kinase concentration used for phosphorylation was increased 50-100-fold, threonine 18 could also be phosphorylated. Phosphorylation of threonine 18 in addition to serine 19 has been shown to result in an increase in the actin-activated MgATPase activity of turkey gizzard smooth muscle HMM when measured at a single actin concentration (6, 7). Fig. 1 shows that this was due to an effect on the K actin (the apparent dissociation constant of HMM for actin measured by a Double-reciprocal Plot of velocity versus actin concentration). There was little difference between the Vmax of serine 19-phosphorylated HMM and that of serine 19 plus threonine 18-phosphorylated HMM. We also studied the effect of threonine 18 phosphorylation on the movement of phosphorylated myosin-coated beads. There was no significant increase in the velocity of myosin-coated beads from turkey gizzard, bovine aorta, and bovine trachea when threonine 18 was phosphorylated by incubation with high concentrations of MLC kinase (Table II).

Protein kinase C can also phosphorylate the 20,000-Da light chain of myosin at several sites (9-11). The velocity was the same for beads coated either with myosin which had been phosphorylated at serine 19 alone by MLC kinase or with myosin which had been phosphorylated with both MLC kinase and protein kinase C (Table III). This was determined for smooth muscle myosin from turkey gizzard, bovine aorta, and bovine trachea and for cytoplasmic myosin from human platelets. If myosin was either unphosphorylated or phosphorylated with only protein kinase C, there was no movement of the beads. In the above experiments, protein kinase C phosphorylation of the myosin which had been previously phosphorylated by MLC kinase (1 mol of P_i/mol of light chain) resulted in the incorporation of an additional 1 mol of P_i/mol of light chain (at sites corresponding to threonine 9

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**Table I**

| Myosin Type       | Velocity (μm/s) | No. of Experiments |
|-------------------|-----------------|--------------------|
| Rabbit skeletal muscle | 1.92 ± 0.072    | 6                  |
| Phosphorylated turkey gizzard | 0.198 ± 0.007 | 10                 |
| Phosphorylated bovine aorta | 0.131 ± 0.021 | 5                  |
| Phosphorylated bovine trachea | 0.117 ± 0.004 | 6                  |
| Phosphorylated human platelet | 0.039 ± 0.004 | 4                  |

* Mean ± S.E.

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**FIG. 1.** Effect of threonine 18 phosphorylation on the actin-activated MgATPase activity of smooth muscle HMM. Mono-phosphorylated (serine 19) or diphostorylated (serine 19, threonine 18) gizzard HMM was assayed for MgATPase activity in the presence of varying concentrations of F-actin. The Vmax and Kactin of monophosphorylated (○) and diphostorylated (●) HMM were 1.05 ± 0.18, 28 μM and 1.17 ± 1, 18 μM, respectively. The conditions were: 10 mM imidazole (pH 7.0), 2 mM MgCl₂, 1 mM ATP, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.2 μM HMM at 25 °C.
TABLE II
Effect of threonine 18 phosphorylation on myosin-coated bead velocity

Effect of protein kinase C phosphorylation on myosin-coated bead velocity

Effect of tropomyosin on myosin-coated bead velocity

TABLE IV
Effect of tropomyosin on myosin-coated bead velocity

FIG. 2. Effect of tropomyosin (TM) on the actin-activated MgATPase activity of phosphorylated smooth muscle HMM. The MgATPase activity of phosphorylated (serine 19) gizzard HMM was assayed either with varying concentrations of F-actin alone or with F-actin-tropomyosin (5:1, molar ratio). The V_{max} and K_{ATPase} of HMM in the presence of actin alone (○) or actin-tropomyosin (□) were 1.9 s^{-1}, 38 μM and 4.7 s^{-1}, 31 μM, respectively. The conditions were: 10 mM imidazole (pH 7.0), 0.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1 mM ATP, 0.2 μM phosphorylated HMM at 25 °C.

Displacement velocity occurs at a tropomyosin concentration of about 0.01 mg/ml. This represents a tropomyosin concentration where the effect is maximal. The half-maximal increase in velocity occurs at a tropomyosin concentration of about 0.01 mg/ml. The data are taken from a representative experiment. There is a significant difference between these two experiments (p < 0.01).

The MgATPase activity of phosphorylated smooth muscle HMM.

Mean ± S.D.

Myosin Phosphorylation state Velocity μm/s
Turkey gizzard Ser-19 0.188 ± 0.028
Ser-19, Thr-18 0.191 ± 0.025
Bovine trachea Ser-19 0.135 ± 0.006
Ser-19, Thr-18 0.133 ± 0.013
Bovine aorta Ser-19 0.107 ± 0.019
Ser-19, Thr-18 0.114 ± 0.029

* Mean ± S.D.

Myosin Phosphorylation by Velocity μm/s
Turkey gizzard MLCK alone 0.198 ± 0.023
MLCK + PKC 0.193 ± 0.028
PKC alone None
Bovine trachea MLCK alone 0.113 ± 0.022
MLCK + PKC 0.109 ± 0.039
PKC alone None
Bovine aorta MLCK alone 0.090 ± 0.013
MLCK + PKC 0.079 ± 0.025
PKC alone None
Human platelet MLCK alone 0.035 ± 0.01
MLCK + PKC 0.034 ± 0.01
PKC alone None

Phosphorylated turkey gizzard

Phosphorylated turkey gizzard

Mean ± S.D.

* Mean ± S.D.

and to serine 1/serine 2). In addition, with platelet myosin there was significant incorporation of 32P into the heavy chain (typically 0.2–0.3 mol of P_i/mol of heavy chain) primarily at a single site (34). The myosins in these experiments were phosphorylated to 1 mol of P_i/mol by MLC kinase or to 1 mol of P_i/mol with MLC kinase plus 1 mol of P_i/mol by protein kinase C. In one experiment with turkey gizzard myosin, the stoichiometry of phosphorylation was 2 mol of P_i/mol of MLC by protein kinase C and 1 mol of P_i/mol by MLC kinase. These myosin-coated beads moved at the same velocity as beads coated with myosin phosphorylated to 1 mol of P_i/mol of MLC by MLC kinase.

Tropomyosin binding to actin has been shown to result in a higher actin-activated MgATPase activity of phosphorylated smooth muscle myosin than when myosin is assayed with actin alone (12). When phosphorylated turkey gizzard HMM was assayed with either pure actin or tropomyosin-actin, it was seen that this increase was due to an effect on the V_{max} with little or no effect on the K_{ATPase} (Fig. 2). When Nitella was dissected in the presence of tropomyosin, the velocity of the beads was about 50% faster than when no tropomyosin was added to the assay (Table IV). Note that the conditions of this assay were different from our standard conditions in that the KCl concentration was 40 mM. This was done in order to facilitate tropomyosin binding to actin. Phosphorylated smooth muscle myosin-coated beads moved somewhat faster at this higher ionic strength than they did under our standard conditions. The absolute velocity of movement of a given myosin was dependent upon conditions of the assay such as temperature, ionic strength, and MgCl₂ concentration.2

DISCUSSION

The Nitella-based in vitro motility assay which was developed by Sheetz, Spudich, and their colleagues (13, 14, 18–21) has been shown to be a quantitative assay to measure the velocity of myosin-coated beads on an organized substratum of actin. Several characteristics of the Nitella system suggest that it is an in vitro correlate of the unloaded shortening velocity of muscle fibers. The velocities of both skeletal muscle myosin and smooth muscle myosin-coated beads agree reasonably well with estimates of the velocity of the sliding of actin filaments past myosin filaments during an unloaded shortening of the respective fibers (13, 35). Also, the velocity

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of myosin-coated beads is largely independent of the myosin concentration added to the beads and is independent of the size of the bead. The unloaded shortening velocity of a muscle fiber is independent of the number of myosin beads contributing to the movement (36).

In this study we have examined the effect of phosphorylation on the movement of smooth muscle myosin- and cytoplasmic myosin-coated beads. The surprising finding is that phosphorylated human platelet myosin-coated beads move at such a low velocity; however, we find that this velocity is characteristic of other vertebrate cytoplasmic myosin-coated beads which we have examined. It is difficult to make a direct comparison between the velocities of particular myosin-coated beads and literature values for the actin-activated MgATPase activities since the MgATPase activity measurement can be so variable depending upon assay conditions and other variables. However, one report (37) shows that phosphorylated platelet myosin and phosphorylated gizzard myosin have similar MgATPase values when measured under identical conditions.

The movements of all of these vertebrate smooth muscle or cytoplasmic myosin-coated beads are totally dependent on phosphorylation of the 20,000-Da light chain under our standard assay conditions. This can be directly demonstrated by the addition of beads coated with unphosphorylated myosin to Nitella. These beads do not move at a measurable rate even when observed for long periods of time. Subsequent addition of a calcium-insensitive proteolytic fragment of MLC kinase (42) to the assay solution results in the movement of all of the beads after a brief lag period which presumably represents the time required to phosphorylate the myosin. The lack of movement of unphosphorylated myosin-coated beads under these conditions is not thought to be due to the lack of myosin binding to the beads for the following reasons. 1) The experiment described above with the calcium-insensitive proteolytic fragment of MLC kinase suggests that myosin is already attached to the beads even though they are immobile. 2) Beads that are not coated with myosin are monodisperse and do not tend to clump or form aggregates. In contrast, beads coated with either phosphorylated or unphosphorylated myosin tend to clump or form multibead aggregates presumably by virtue of myosin filament cross-linking of several beads. 3) Electron micrographs of beads coated with either phosphorylated or unphosphorylated smooth muscle myosin which were diluted into the buffer used for the Nitella assay while being prepared for electron microscopy revealed the presence of filaments in both cases (20).

The 20,000-Da light chain of smooth muscle myosin can be phosphorylated at several other residues, and these phosphorylations have been shown to affect the actin-activated MgATPase activity. Threonine 18 can be phosphorylated by MLC kinase in addition to serine 19 if large amounts of MLC kinase are used. It has been shown that this phosphorylation resulted in an increase in the actin-activated MgATPase activity of smooth muscle HMM (6-8). Our results indicate that this is due to an increase in the apparent affinity of HMM for actin (i.e. a decrease in the $K_{ATPase}$) with less effect on the $V_{max}$ in general, we find a smaller increase in activity due to threonine 18 phosphorylation than was described by Ikebe and Hartshorne (6). We also find that there is no effect of threonine 18 phosphorylation on the movement of smooth muscle myosin-coated beads. Platelet myosin was not easily diphosphorylated by MLC kinase and was not tested in this assay.

It has been shown that protein kinase C phosphorylation of smooth muscle HMM (at threonine 9 and serine 1 or 2) results in a decrease in the actin-activated MgATPase activity through an effect on the $K_{ATPase}$ with little effect on the $V_{max}$ (9, 11). Our results indicated that this phosphorylation has no effect on the movement of either smooth muscle myosin- or human platelet myosin-coated beads. Protein kinase C phosphorylates platelet myosin on the heavy chain as well as the 20,000-Da light chain, and approximately 0.3 mol of P/ mol of heavy chain was incorporated into a single major serine-containing site in the experiments (34). Thus it appears that this level of heavy chain phosphorylation of platelet myosin by protein kinase C does not affect motility.

Tropomyosin binding to actin has also been shown to increase the actin-activated MgATPase activity of smooth muscle myosin (12), and our data show that with turkey gizzard HMM this is due to a direct effect on the $V_{max}$ with little effect on the $K_{ATPase}$. Tropomyosin addition to the in vitro motility assay buffer results in a large increase in the velocity of smooth muscle myosin-coated beads. There is no evidence for the existence of tropomyosin in Nitella. Another algal system (Chara), which has similar actin filaments, can bind tropomin-tropomyosin complexes to give a calcium-dependent motility system (38).

While the phosphorylation of smooth muscle myosin at serine 19 by MLC kinase in vitro has been shown to result in a large increase in the actin-activated MgATPase activity, the role of this phosphorylation in vivo is not so simple. Marked phosphorylation of the 20,000-Da light chain of myosin precedes tension development following the addition of an agonist or the electrical stimulation of smooth muscle fibers (1-3). During the phase of active tension production the unloaded shortening velocity of the muscle is high. Smooth muscle fibers can maintain tension for extended periods of time, and during the tension maintenance phase of the contraction the unloaded shortening velocity declines substantially. This condition was termed "latch" by Murphy and his colleagues and is characterized by tension production by slowly cycling crossbridges (43). In some smooth muscle fibers under some conditions the phosphorylation level of the 20,000-Da light chain declines from its early peak values to very low levels during the latch condition (43). Thus the level of myosin light chain phosphorylation does not always correlate directly with tension in intact smooth muscle fibers.

Most studies of muscle contraction have only examined the total incorporation of phosphate into the light chain, and there is little information available on the sites of phosphorylation. Several recent reports suggest that the light chain can be diphosphorylated in smooth muscle fibers (39-41). Kamm et al. (40) found that following carbachol treatment of tracheal smooth muscle, up to about 10% of the light chain could be diphosphorylated, whereas 60% of the light chain was monophosphorylated at the MLC kinase site. The site of the diphosphorylated light chain was shown to be different from the sites phosphorylated by protein kinase C in vitro. In the case of glyc erinated porcine carotid artery muscle fibers, the diphosphorylated light chain was thought to be the result of phosphorylation of both serine 19 and threonine 18 presumably by MLC kinase (41). It is not yet known what the functional consequences of this extra phosphorylation are in vivo, but it has been suggested that it does not affect either the level of steady-state tension or the unloaded shortening velocity of the skinned fiber preparation. This later observation is consistent with our finding that phosphorylation of threonine 18 in addition to serine 19 does not lead to an increase in the velocity of myosin-coated beads.

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