Spatial Pattern of Myosin Phosphorylation in Contracting Smooth Muscle Cells: Evidence for Contractile Zones

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Abstract. We have purified a polyclonal antibody by affinity chromatography which binds specifically to the phosphorylated form of the regulatory light chain (M, = 20,000) of smooth muscle myosin. This antibody does not stain relaxed, permeabilized smooth muscle cells isolated from guinea pig taenia coli. However, when these cells were stimulated to contract with CaCl₂ (100 μM) and ATP (1 mM), the immunofluorescence staining was localized in a series of transverse bands. This distribution of activated myosin appears to reflect an underlying structural organization of the smooth muscle cell cytoskeleton into mechanically coupled contractile zones.

Smooth muscle has contractile properties which differ from those of striated muscle; in general, smooth muscle cells contract more slowly, they can shorten to a much greater extent, and can maintain contraction with a smaller consumption of ATP. However, very little is known about the functional organization of the contractile components of these cells. Smooth muscle cells have no obvious direct equivalent of the striated muscle sarcomere, although actin and myosin filaments are aligned approximately in parallel (4). A certain amount of controversy has surrounded the stability of the myosin filaments in these cells. Although myosin filaments have been detected in smooth muscle cells under both relaxed and contracting conditions (29), both in vitro (6) and in vivo (11, 12), data now indicate that the organization of myosin in these cells may be dynamic, as it appears to be in motile nonmuscle cells (22). At the biochemical level, it is well established that phosphorylation of the 20-kD regulatory myosin light chain (LC20) both controls the force-generating interaction of myosin with actin and promotes the self-assembly of myosin monomers to the filamentous form that is thought to be obligatory for contraction (1, 16, 23, 30, 32).

As a result, one would expect the regions of contractile activity within a cell to contain phosphorylated myosin. Accordingly, we have developed an antibody that specifically recognizes the phosphorylated form of myosin in order to identify contractile regions by immunofluorescence. In this paper we describe this antibody and its use to reveal the organization of activated myosin in contracting smooth muscle cells. Our data suggest a new level of organization; the presence of zones along the cell where phosphorylated myosin becomes concentrated during contraction.

Materials and Methods

Myosin light chain peptide 6-23 (kindly provided by Dr. B. Kemp, University of Melbourne, Melbourne, Australia) was phosphorylated enzymically (16, 20) and the phosphopeptide purified on a CM52 column. Phosphopeptide was coupled to keyhole limpet hemocyanin (Calbiochem-Behring Corp., La Jolla, CA) at a mass ratio of 0.5:1 using 0.04% glutaraldehyde in the presence of 0.1% SDS. The peptide, phosphopeptide, LC20, and thio-phosphorylated LC20 (prepared as in reference 16) were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) using the manufacturer's recommended procedure. Antiserum was obtained from a rabbit which had been immunized by subcutaneous injection of 200 μg of the keyhole limpet hemocyanin–phosphopeptide 6-23 conjugate in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, MI) containing sodium pyrophosphate (20 mM) as a phosphatase inhibitor. The response was boosted with unconjugated phosphopeptide 6-23 (50 μg) in incomplete Freund's adjuvant and sodium pyrophosphate (20 mM) at ~2-mo intervals.

The crude antiserum was affinity purified in four steps. (a) Serum was loaded on to a column containing phosphopeptide 6-23 coupled to Sepharose, and washed with a column buffer consisting of 10 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl, 2 mM sodium pyrophosphate, 5 mM NaN₃. Bound antibody was removed with an elution buffer (0.2 M glycine buffer, pH 2.7, 0.2 mM sodium pyrophosphate, 3 mM NaCl) and the eluate was immediately neutralized with Tris base. Fractions containing antibody to phosphopeptide 6-23 were identified by ELISA and pooled. (b) The eluted antibody was passed down a column containing peptide 6-23 coupled to Sepharose. (c) Antibody that failed to bind was passed down a column containing LC20 coupled to Sepharose. (d) Antibody that failed to bind was loaded onto a column containing thio-phosphorylated LC20 coupled to Sepharose, and washed with column buffer. Material that bound to the column was washed off in elution buffer and immediately neutralized. Fractions containing antibody to phosphopeptide 6-23 were pooled and stored at ~20°C.

Smooth muscle cells were isolated using a procedure based on reference 25. Taenia coli muscles were dissected from a freshly killed guinea pig and immediately placed in a buffered salts solution (BSS) comprising 137 mM NaCl, 5 mM KCl, 20 mM Heps buffer, pH 7.4, 1 mM MgCl₂, 1 mg/ml
glucose, 1 mg/ml BSA, 0.1 mg/ml soybean trypsin inhibitor. The muscle strips were tied to pieces of plastic and digested with 1 mg/ml collagenase (type CLS II; Worthington Biochemical Corp., Freehold, NJ) for 45 min at 37°C in BSS containing 50 μM CaCl₂. The muscles were rinsed in BSS containing 0.5 mM DTT but no added calcium, and finally given gentle mechanical disruption in BSS containing 20 μM EGTA. Free cells were washed into BSS containing no serum albumin or trypsin inhibitor by gentle centrifugation (600 rpm for 3 min).

Cells were stimulated to contract by adding an equal volume of a contraction solution containing 2 mM ATP, 0.2 mM CaCl₂, and 0.2% Triton X-100 in BSS (modified as indicated for the experiments of Tables I and II). These cells typically shortened to between 25 and 40% of their starting length, with a t½ for contraction of 30-60 s at 20°C. Contraction was terminated by rapidly fixing the slide with 3.7% formaldehyde in 150 mM NaCl, 2 mM sodium pyrophosphate, 10 mM sodium phosphate buffer, pH 7.0. After 2-5 min, each slide was transferred for overnight fixation at 4°C to a solution containing 0.5% glutaraldehyde in 150 mM NaCl, 2 mM sodium pyrophosphate, 10 mM sodium citrate buffer, pH 5.0 (the low pH prevented LC20 from being lost before being cross-linked into the myosin).

The cells were treated with 0.2% Triton X-100 for 5 min, then given three successive incubations in freshly prepared 10 mM NaBH₄ (34). Indirect immunofluorescence labeling (33) was carried out in 150 mM NaCl, 2 mM sodium pyrophosphate, 10 mM sodium phosphate buffer, pH 7.0, 10 mg/ml BSA. The affinity-purified antiserum was used at a concentration of 80 μg/ml protein, with a fluorescein-conjugated secondary antibody. Monoclonal antibody BM3 to myosin heavy chain has been previously described (5) and was used with a rhodamine-conjugated secondary antibody.

Measurement of antibody reactivity by ELISA was carried out according to reference 15 using microtiter plates coated with antigen at ~25 pmol/well. A peroxidase-coupled secondary antibody was used.

PAGE in the presence of SDS was carried out according to reference 18. Transfer of proteins to nitrocellulose was carried out electrophoretically (31). The nitrocellulose blot was fixed for 10 min with 2.5% glutaraldehyde, rinsed, and incubated with 50 mM NaBH₄ to reduce unreacted aldehyde groups (LC20 is extremely soluble, and was slowly leached from the nitrocellulose if not fixed). Antibody incubation and washes were carried out in 150 mM NaCl, 2 mM sodium pyrophosphate, 10 mM sodium phosphate buffer, pH 7.0, 0.05% Tween 20, and a peroxidase-coupled secondary antibody was used. Parallel strips of nitrocellulose were stained for protein using Amido black.

**Results**

The affinity-purified antibody raised against phosphopeptide 6-23 bound to both phosphorylated peptide 6-23 and phos-

**Table I. The Effect of Nucleotides on Myosin Light Chain Phosphorylation, Detected by Immunofluorescence**

| Condition          | Experiment 1 | Experiment 2 | Experiment 3 |
|--------------------|--------------|--------------|--------------|
| ATP*               | 100          | 100          | 100          |
| ATPγS              | 98           | 76           | 87           |
| AMP-PNP            | 13           | 17           | 15           |
| ADP                | 17           | 18           | --           |
| No nucleotide      | 11           | 34           | 29           |
| ATP + 20 μM CAMP   | 85           | 93           | 96           |
| ATP + 2 mM EGTA    | 2            | 0            | 0            |

Smooth muscle cells were treated with nucleotide at a final concentration of 1 mM (unless shown otherwise) in the presence of 0.1 mM CaCl₂ and 0.1% Triton X-100, and the proportion of cells showing immunofluorescence due to the antibody to phosphorylated LC20 was determined. On each slide, morphologically intact smooth muscle cells were identified using phase-contrast optics, and then scored for antibody staining after switching to epifluorescence illumination. For each condition >200 cells were examined. The antibody was used at a concentration of 80 μg/ml protein, with a fluorescein-conjugated secondary antibody. Monoclonal antibody BM3 to myosin heavy chain has been previously described (5) and was used with a rhodamine-conjugated secondary antibody.

**Table II. The Effect of Calcium Ion Concentration on the Phosphorylation of Myosin Light Chain, Detected by Immunofluorescence**

| Condition                                      | Result |
|-----------------------------------------------|--------|
| 100 μM Ca²⁺                                   | 100*   |
| 1 μM Ca²⁺                                     | 79     |
| 50 nM Ca²⁺                                    | 6      |
| <5 nM Ca²⁺                                    | 0      |
| 100 μM Ca²⁺ + 10 μM trifluoperazine           | 49     |
| 100 μM Ca²⁺ + 100 μM trifluoperazine          | 24     |

Smooth muscle cells were treated with Ca²⁺ and trifluoperazine at the concentrations shown in the presence of 1 mM ATP and 0.1% Triton X-100. The lower calcium ion concentrations were obtained using EGTA-CaCl₂ buffers (14) at 2 mM.

The proportion of cells showing immunofluorescence was normalized as in Table I, with the reference condition corresponding to 227 cells staining out of 435 examined.

Phosphorylated myosin but not to unphosphorylated antigen (Fig. 1). In other experiments (not shown), the antibody showed a similar specificity for the phosphorylated form of the isolated LC20 by ELISA, and stained only the bands corresponding to phosphorylated LC20 on a nitrocellulose blot of a urea–glycerol gel (21) of phosphorylated myosin. The antibody bound to a single protein band that comigrated with LC20 in samples of contracted smooth muscle cells analyzed by SDS-PAGE (Fig. 2).

The antibody specific for phosphorylated LC20 stains isolated smooth muscle cells from guinea pig *taenia coli* that have been permeabilized and induced to contract with CaCl₂ and ATP (Fig. 3). Typically, ~50-60% of the cells in any preparation responded to this contracting solution and stained with the antibody. Smooth muscle cells that failed to contract did not stain with the antibody; neither did unstimulated cells. All cells, whether or not they contracted, contained myosin as detected using antibody BM3 (5), which is a monoclonal antibody that reacts against the COOH-terminal end of nonmuscle or smooth muscle myosin heavy chains.

Immunofluorescence of stimulated cells followed the known ability of various ATP analogues to act as substrates for myosin light chain kinase (Table I). Myosin light chain kinase is known to accept adenosine-5'-O-(3-thiotriphosphate) as a substrate, but not adenylyl-imidodiphosphate or ADP (2). The low level of staining seen with the poor kinase substrates is probably due to endogenous cellular ATP, since a similar level of staining was seen in the absence of any added nucleotide.

The calcium ion concentration necessary for activation as assayed by antibody staining was found to be of the order of 1 μM (Table II). Staining was inhibited by trifluoperazine, at the concentrations which inhibit calmodulin-dependent activation of myosin light chain kinase in vitro (*K₅ = 6 μM; reference 36).

These results show that the amount of immunofluorescence staining is modulated by agents which alter LC20 phosphorylation by myosin light chain kinase. It is interesting that cAMP has no effect in this system (Table I) since in vitro evidence has indicated that a CAMP-dependent protein kinase could down-regulate myosin light chain kinase (3). In other experiments GMP and tetradecanoyl phorbol acetate (an activator of protein kinase C) were also found not to...
inhibit myosin phosphorylation. Neither did any of these reagents cause any significant phosphorylation of LC20 as detected with the antibody when the calcium ion concentration was buffered to 50 nM (data not shown).

The immunofluorescence staining of contracted smooth muscle cells appeared cytoplasmic with a periodic distribution (Fig. 4). In some cases this amounted to an undulating intensity of staining along the length of the cell (e.g., Fig. 4, A and B), while in others there appeared to be discrete bands. In favorable cases the fluorescent staining could be resolved into longitudinal elements (e.g., Fig. 4 B). In some cells staining appeared concentrated towards the edge of the cell, though still with a banded distribution (Fig. 4 H); this pattern was characteristically observed in the shortest cells so that we are unable to rule out the possibility that, in these cells, the density of cytoskeletal components is limiting the access of antibody into the middle region of the cell.

In a number of cases the positions of regions containing phosphorylated myosin correlated with bulges in the profile of the cell (e.g., Fig. 4, C, G, and H), possibly indicating that contractile elements were attached to the cell membrane on each side of the staining region. The banded appearance was not a simple consequence of viewing a greater thickness of cytoplasm and hence correspondingly more myosin in those regions. The bands were still apparent when cells were examined using a scanning confocal fluorescence microscope (35) that images optical sections with a depth of only ~0.7 μm (data not shown). The localization of total myosin (using monoclonal antibody BM3) showed a much less marked banding pattern when it was examined in double-label experiments (Fig. 5), indicating that this pattern is specifically that of phosphorylated myosin. In the case of total myosin, the slightly nonuniform distribution is probably explained by the enhanced solubility of unphosphorylated myosin (23), which means that in the permeabilized cells used here myosin would preferentially dissociate from the regions between bands of phosphorylated myosin.

Statistical analysis (Fig. 6) indicates that the relative positions of fluorescent bands were consistent from cell to cell. In spite of the fact that the lengths of individual half cells ranged from 15 to 125 μm, seven positions relative to the axial length appeared to be favored as locations of phosphorylated myosin (Fig. 6 B). However, the peaks on the histogram are not completely separated, raising the possibil-
ity that there was a mixture of subpopulations. Further division of the sample showed that ~50% of the half cells examined had band positions which formed a seven peak histogram (Fig. 6 C), while a further 40% had an eight peak pattern (Fig. 6 D). The remaining few half cells probably had a six-band pattern (data not shown).

Discussion

The antibody-staining pattern we observe suggests that phosphorylated myosin is concentrated in a number of "contractile zones" along the length of a contracting smooth muscle cell, typically 14-16 zones per cell. What might be the cellular basis of this pattern?

The most likely explanation is that the pattern reflects an underlying feature of the cytoskeleton, consistently present in smooth muscle cells. In Triton-permeabilized cells it cannot be a consequence of a spatial periodicity in the calcium signal, which would be uniform. Neither do we think it likely to be due to the distribution of myosin light chain kinase in the cell. Although there is no direct information on the intracellular localization of the kinase in smooth muscle cells, it binds to both myosin filaments and actin filaments (7, 24) which suggests that it will be fairly uniformly distributed, as it is in stress fibers of nonmuscle cells (8, 13).

A possible explanation for the observed localization of immunofluorescence in discrete zones along the cell length is that there are regions where myosin translocation along actin filaments is permitted, and others where movement is prevented. Activated myosin filaments will move with respect to actin, from the pointed end of an actin filament towards the barbed end, as long as there is no obstacle to further binding...
of myosin heads. Thus, phosphorylated myosin will tend to accumulate on the pointed-end side of such an obstacle and become depleted from the barbed-end side. This sort of block to myosin translocation might exist at the dense bodies (4) where actin filaments attach to the cell membrane or to each other, or it might be due to the presence of proteins which sterically hinder myosin binding. It will clearly be important to see if the band positions correlate with a transverse register of other actin-binding proteins. Although previous work has not suggested any obvious periodicity of ultrastructural organization along the length of cells (4, 10, 19, 26, 28), it will also be interesting to reexamine contracting smooth muscle cells by electron microscopy.

This is the first demonstration that myosin is organized into contractile zones in smooth muscle cells. There have been occasional reports of dense-staining bands in histological preparations of smooth muscle (17), although these have most often been assumed to be artefactual. It must be stressed that in the experiments reported here, a zone pattern for phosphorylated myosin was not normally accompanied by dark bands when viewed under phase contrast. Only after long contraction times or in relatively acid buffers (pH < 6.5) did we see a banding pattern in phase contrast. At this pH myosin filaments are stabilized against disassembly in the presence of Mg-ATP (23, 30); hence, if further translocation along actin filaments was blocked in certain regions, myosin would accumulate in unusually large concentrations at those locations.

The amount of phosphorylation in individual contractile zones can vary without altering the relative locations of the zones. For most of the half cells analyzed in Fig. 6, fewer than seven positively staining regions were scored, and yet...
their positions fell into one of the patterns illustrated in Fig. 6, C and D. Some of the seven or eight contractile zones in each half cell were “silent”, while the rest retained their expected position. The inference is that the zones are mechanically coupled such that they all shorten together even when the myosin-based forces causing shortening are present in only some of the zones. The structural basis of zone coupling can only be a matter for speculation at present. It may be relevant that Small’s laboratory (9, 27) have demonstrated the presence of longitudinal fibrillar domains in the smooth muscle cell cytoskeleton that seem to have a purely mechanical function (they contain actin, filamin, and desmin, but no myosin). These are quite distinct from the contractile domains which contain actin and myosin.

It is not only in smooth muscle but also in other motile cells that myosin light chain phosphorylation both controls force generation and promotes the self-assembly of myosin monomers onto filaments. We anticipate that the antibody described here will prove to be a useful tool for the localization of actively contractile regions of nonmuscle cells undergoing motile processes such as ameboid locomotion, stress fiber formation and breakdown, and cytokinesis.

Figure 6. Analysis of the positions of maximum immunofluorescence intensity along the length of smooth muscle cells. Photographic enlargements of contracting cells were analyzed using a microcomputer with input from a digitizing tablet (kindly made available by Dr. C. Stolinski, St. Mary’s Hospital Medical School, London). For each cell, a line was constructed along the axis of the cell and the distance of the center of each band from the nuclear margin along this line was measured for both halves of the cell. The histograms are plotted as a function of the position of each band expressed as (A) its distance in μm from the nucleus, or (B, C, and D) the same distance expressed as a percentage of the distance to the cell tip. A and B show the complete set of 65 half cells (276 bands), while C and D show selected subpopulations of 33 and 26 half cells, respectively.

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