An Increase or a Decrease in Myosin II Phosphorylation Inhibits Macrophage Motility

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Abstract. Myosin II purified from mammalian non-muscle cells is phosphorylated on the 20-kD light chain subunit (MLC20) by the Ca2+/calmodulin-dependent enzyme myosin light chain kinase (MLCK). The importance of MLC20 phosphorylation in regulating cell motility was investigated by introducing either antibodies to MLCK (MK-Ab) or a Ca2+/calmodulin-independent, constitutively active form of MLCK (MK-) into macrophages. The effects of these proteins on cell motility were then determined using a quantitative chemotaxis assay. Chemotaxis is significantly diminished in macrophages containing MK-Ab compared to macrophages containing control antibodies. Moreover, there is an inverse relationship between the number of cells that migrate and the amount of MK-Ab introduced into cells. Interestingly, there is also an inverse relationship between the number of cells that migrate and the amount of MK- introduced into cells. Other experiments demonstrated that MK-Ab decreased intracellular MLC20 phosphorylation while MK- increased MLC20 phosphorylation. MK- also increased the amount of myosin associated with the cytoskeleton. These data demonstrate that the regulation of MLCK is an important aspect of cell motility and suggest that MLC20 phosphorylation must be maintained within narrow limits during translational motility by mammalian cells.

CELLULAR locomotion by mammalian cells is essential for embryogenesis, cell-mediated killing (4), and the formation of metastatic colonies by cancer cells. Cell motility is a complex process that requires the coordinated regulation and the interaction of numerous reactions. ATP hydrolysis by actin and myosin II and myosin II polymerization/depolymerization are thought to be among the reactions involved in mediating translational motility (2, 23). In mammalian nonmuscle cells, both ATP hydrolysis (3, 30, 31) and filament formation (10, 27) by myosin II are regulated by phosphorylation of the 20-kD light chain of myosin (MLC20) by myosin light chain kinase (MLCK) (2). Therefore, MLCK and MLC20 phosphorylation are thought to play critical roles in regulating cell motility.

However, the role of filamentous myosin (myosin II) in cell motility is unclear. Experiments on the slime mold Dicystostelium discoideum have questioned the importance of filamentous myosin in cell motility (14, 22). Dicystostelium contains two myosins designated myosin I and myosin II (24). Myosin I is a single-headed myosin with a short heavy chain that does not form filaments (24). Myosin I associates with lipids (1) and has been localized in the leading edges of lamellipodia of migrating Dicystostelium ameoba (17). Myosin II is similar to mammalian muscle and nonmuscle myosins in that it has two globular heads, a coiled-coiled tail, and an ability to form filaments. In contrast to mammalian nonmuscle and smooth muscle myosin II, ATP hydrolysis and filament formation by Dicystostelium myosin II are regulated by both heavy chain and light chain phosphorylation (24). Interestingly, Dicystostelium in which myosin II heavy chain expression has been genetically manipulated are still capable of locomotion (14, 22). These data have questioned the importance of myosin II and its regulation by phosphorylation in cell motility.

On the other hand, experiments on vertebrate cells have generally supported a role for myosin II and MLCK in cell motility. The addition of affinity-purified antibodies to MLCK (MK-Ab) to mouse fibroblasts with permeabilized membranes inhibited lamellipodial retraction (20), while the addition of trypsinized, constitutively active MLCK (MK-) to permeabilized chick embryo fibroblasts resulted in MLC20 phosphorylation and lamellipodial contraction (6). Although lamellipodial retraction is an important aspect of translational motility, the role of MLCK in the motile process was not investigated in these fibroblast preparations because cells with permeabilized plasma membranes are incapable of translational motility and/or chemotaxis.

Therefore, we have investigated the role of myosin II and its regulation by phosphorylation by MLCK in mediating mammalian cell motility. In order to do so, MK-Ab and MK-, introduced into physiologically responsive macrophages with intact plasma membranes, were used to manipulate intracellular MLCK activity. The effects of increasing or
Materials and Methods

Proteins

Control antibodies (C-AbS) were purified from normal rabbit serum by affinity chromatography on a protein A-Sepharose column. MK-AbS were produced and affinity purified as described (11). MK-Ab was purified by digesting purified chicken gizzard MLCK with trypsin as previously described (12). BSA was also digested with trypsin at a similar protein/trypsin ratio (40:1), and used as a control in the MK-Ab experiments. Trypsin was inactivated by adding a 100-fold excess of soybean trypsin inhibitor to the BSA and MLCK digests.

Electroinjection

Electroinjection (single discharge) was performed at 0°C and at different field strengths by changing the voltage setting of a Bio-Rad Laboratories (Richmond, CA) Gene Pulser (capacitance = 15 μF, interelectrode distance of 0.4 cm). The cell suspension was removed from the chamber and incubated for 15 min at 0°C. The macrophages were collected by centrifugation and resuspended in Gey's balanced salt solution (Grand Island Biological Company, Grand Island, NY) containing 1% BSA at 2 x 10^6 cells/ml. In all experiments, viability was assessed after electroporation by adding trypan blue (0.4%) to an aliquot of cells and quantifying the percentage of cells that excluded dye after 5–15 min at 23°C.

Chemotaxis Assays

Cell motility was quantitated by performing directed chemotaxis assays as previously described (13). Briefly, 10^5 cells were loaded into each of the top chambers of a 48-well microchemotaxis chamber (Neuro Probe, Inc., Cabin John, MD). The lower chambers contained endotoxin-activated rat serum as a chemoattractant (14). The macrophages migrated toward the chemoattractant passing through a polycarbonate-pyrolydine-coated 5-μm pore size filter. After a 90-min incubation at 37°C, the unMigration macrophages were scraped off the topside of the filter. The cells that migrated through the filter and attached to the underside were fixed, stained, and quantified. Each condition was run in triplicate and the cells in five oil immersion fields in each triplicate were counted and averaged to determine the extent of migration.

Quantitation of Protein Incorporation

Macrophages electroinjected in the presence of 2.5 mg/ml rhodamine-labeled BSA were washed three times in Gey's solution. The cells were resuspended at a concentration of 2 x 10^5 cells/ml in Gey's solution containing 1% BSA and fluorescence was quantitated using a Perkin-Elmer model LS-5 fluorescence spectrophotometer (ex/em 570/589 nm). Fluorescence from control cells that were not electroinjected but that were treated identically to electroinjected cells in every other way were subtracted from the experimental data. Protein incorporation was calculated based on a standard curve of fluorescence using known amounts of rhodamine-labeled BSA.

Flow Cytometry

The uniformity of protein incorporation was investigated by performing fluorescence-activated cell sorting. Macrophages were either incubated or electroinjected (700 V/cm) in the presence of 2.5 mg/ml fluorescein-labeled BSA. The cells were washed twice and resuspended in Gey's solution containing 1% BSA. Flow cytometry was performed with a Coulter Corporation (Hialeah, FL) Epics V flow cytometer with a 256-channel analyzer interfaced with multi-parameter data acquisition and display computer system. 5-W argon ion laser (Coherent, Inc., Palo Alto, CA) emitting at 488 nm was operated at 260 mW power. Fluorescence was recorded through a log amplifier on a gated population of cells, based upon forward angle and 90° light-scatter properties that represent the main population of cells, thereby eliminating any fluorescence readings from cell debris or cell aggregates. Each analysis was performed on 10,000 cells.

Inhibition of Macrophage MLCK Activity by MK-Ab

Macrophages (10^6 cells) were extracted by freeze/thawing in 0.2 ml of 0.34 M sucrose, 10 mM EDTA, 10 mM DTt, 10 mM glycerophosphate, 10 mM Tris, pH 7.5, 2 mg/1 leupeptin and pepstatin, 100 μg/ml soybean trypsin inhibitor, 180 ng/ml phenylmethylsulfonyl fluoride, and 1% NP-40. After centrifugation at 50,000 g for 10 min, the supernatant was made 10 mM MgATP and fractionated by adding solid (NH4)2SO4. The protein that precipitated between 35 and 60% saturation, which contained most of the myosin light chain kinase activity, was collected by centrifugation, resuspended, and dialyzed against 0.3 M NaCl, 1 mM EDTA, 20 mM MOPS, pH 7.2. Approximately 50 μg of the macrophage proteins were preincubated with 16.5 μg of MK-Ab or C-Ab for 5 min at 0°C. The reaction mixture contained (final concentrations) 0.3 mM CaCl2, 0.1 μM calmodulin, 10 mM MgCl2, 10 μM purified chicken gizzard MLCKo, 100 μM γ-32PATP in a 100-μl reaction volume. Aliquots from the reaction mixture were acid precipitated at defined times and protein-bound cpm were quantitated. The assays were linear with respect to time. Nonspecific phosphorylation, determined by performing kinase assays in the absence of exogenous MLCK, was subtracted from the phosphorylation data.

Western Blot Analysis

Macrophage (10^6 cells) and chick embryo fibroblasts (10^5 cells) were extracted by freeze/thawing as described above. Approximately 100 μg of protein from each cell extract were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose sheets were then processed using MK-Ab (4 μg/ml) as the primary antibody and peroxidase-labeled, goat antirabbit antibody as the secondary antibody. The color reaction was developed using 0.05% 4-chloro-1-naphthol as the substrate.

Determination of Myosin II Distribution

Cytoskeletons from macrophages were prepared as previously described (16). Briefly, an equal volume of ice-cold extraction buffer (2% Triton X-100, 40 mM sodium pyrophosphate, 20 mM potassium phosphate, 10 mM sodium molybdate, 2 mM N-ethylmaleimide, 2 mM EGTA, pH 7.4) was added to macrophages suspended in Gey's balanced salt solution. The insoluble cytoskeletons were immediately isolated by centrifugation at 8730 g for 4 min. The soluble proteins in the supernatant were acetone precipitated and the pellet was rinsed with a 1:1 dilution of the extraction buffer and Gey's balanced salt solution, without resuspension, and collected by centrifugation. The cytoskeletal proteins and the acetone-precipitated cytosolic proteins were solubilized by boiling in SDS-sample buffer containing 5% 2-mercaptoethanol (vol/vol). Approximately 100 μg of protein from each fraction were separated by SDS-PAGE on a 5–20% gradient slab gel. The proteins were transferred to 0.2-μm pore-size nitrocellulose paper at 100 mAmp for 4 h in 20% methanol, 25 mM glycine, pH 8.3, using a transblot apparatus (Bio-Rad Laboratories). These conditions permit quantitative transfer of the myosin heavy chains without loss of the myosin light chains (see below). After transfer, the nitrocellulose sheets were processed as Western blots using rabbit antiplatelet myosin II antibodies and peroxidase-labeled, goat antirabbit antibodies. Antibody binding was visualized using 4-chloro-1-naphthol as a substrate and a band corresponding to an Mr = 204,000, the apparent molecular weight of myosin II heavy chain, developed in each lane. The amount of dye in each of these bands was quantitated by scanning each band with a densitometer (Joyce-Loebl Ltd., Gateshead, England) in the reflectance mode. The area defined by the densitometer tracing, which was a function of the dimensions and the intensity of each band, was taken as a measure of the amount of myosin II heavy chain in each fraction. The amount of myosin II heavy chain in the cytoskeleton was then expressed as the percent total myosin II heavy chain in the cytoskeletal and soluble fractions by dividing the area of the cytoskeletal band by the sum of the areas of the cytoskeletal and soluble fractions.

An important aspect of these experiments is establishing that the myosin subunits are transferred quantitatively to the nitrocellulose sheets. We investigated the transfer of heavy chains by transferring the same gel twice using the conditions described above. When the blots were developed using rabbit antibodies to platelet II myosin and peroxidase-labeled secondary antibodies, most of the myosin II heavy chain was found on the blot after the first transfer.
transfer and very little (<5%) of the heavy chain was detected on the blot from the second transfer. Transfer of MLC20 was investigated by separating two sets of molecular weight standards and 50, 100, and 200 ng of purified, 32P-labeled MLC20 by SDS-PAGE on a 5-20% acrylamide gradient gel. One set of proteins was stained and destained. The other set of proteins was transferred to nitrocellulose as described above, except that two 0.2-µm pore size nitrocellulose sheets were placed in series on the anodic side of the gel during the transfer. After transfer, the regions of the stained gel and the two nitrocellulose sheets containing MLC20 were excised and the radioactivity was quantitated by liquid scintillation counting. The data demonstrated that 95% of the CPM applied to each lane, as indicated by the CPM in each lane of the untransferred; stained gel, was bound to the nitrocellulose sheet adjacent to the gel. Only background levels of radioactivity were detected on the more distal nitrocellulose sheet. Based on these data, we believe that we are quantitatively transferring myosin II heavy chains without loss of the light chains.

**MLC20 Phosphorylation**

Macrophages (3 × 10⁶ cells) were electroinjected with MK-, trypsin-treated BSA, MK-Ab, or C-Ab at a concentration of 2.5 mg/ml at 700 V/cm and 15 µl. The cells were collected, resuspended, and incubated in 0.5 ml of phosphate-free Gey's buffer containing 0.5 mCi of 32P for 60 min at 37°C. Cells injected with MK-Ab and C-Ab were then treated with 1 µM okadaic acid for 10 min. At 70 min, all cells were extracted by freeze/thawing and the myosin was immunoprecipitated using antibodies to platelet myosin II as previously described (25). The immunoprecipitated proteins were separated by SDS-PAGE (5-20% acrylamide separating gel) and transferred to nitrocellulose as described above. The nitrocellulose sheets were then processed as Western blots and the band representing the myosin II heavy chain in each lane was quantitated densitometrically as described in the previous paragraph. The area defined by the densitometer tracing was taken as a representation of the myosin II heavy chain in each immunoprecipitate. The blot was exposed to an X-ray film and the region representing MLC20 in each lane was excised and the radioactivity was quantitated by liquid scintillation counting. The CPM were divided by the area representing the heavy chains from the densitometer tracing to correct for the amount of myosin II in each immunoprecipitate. The CPM/unit area for MK-Ab and MK- were then divided by the CPM/unit area for C-Ab and BSA, respectively, to determine the relative change in MLC20 phosphorylation.

**Results**

A modification of the voltage discharge technique, called electroinjection (7, 32, 35), was used to introduce proteins into macrophages. Experiments that were performed to characterize the effect of electroinjecting macrophages are described in Fig. 1. Fig. 1A illustrates the effect of field strength on macrophage viability assessed by two different methods. Viability, as judged by trypan blue exclusion, remained high over all voltages tested. However, chemotaxis diminished at field strengths >900 V/cm. The relationship between protein loading and field strength was also assessed. The data in Fig. 1B demonstrate that protein incorporation increased as the field strength increased. Flow cytometry was also performed on cells loaded with FITC-BSA at 700 V/cm (Fig. 1C). These experiments demonstrated a single population of labeled cells that contained more fluorescence than cells merely incubated with the FITC-BSA. Since...
significant amounts of protein could be loaded relatively uniformly into cells and the number of cells that migrated remained high up to 900 V/cm, we used 700 V/cm and 15 μF as our standard conditions for electroinjecting macrophages (see ref. 32 for a more complete characterization of the electroinjection technique).

Figure 2. Interaction of MK-Ab with macrophage MLCK. (left panel) Macrophage MLCK was concentrated by ammonium sulfate fractionation (Materials and Methods). The effect of preincubating aliquots of this MLCK fraction with C-Ab or MK-Ab on MLC20 phosphorylation was then determined as described in Materials and Methods. The data demonstrate inhibition of 3P incorporation by MK-Ab. (right panel) Western blot analysis of macrophage (M) and chick embryo fibroblasts (CF) proteins. Approximately 100 μg of macrophage and fibroblast proteins was separated by SDS-PAGE, transferred to nitrocellulose, and probed with MK-Ab as described in Materials and Methods. The most intensely staining bands in the macrophage and fibroblast extracts had an M, = 130,000 and comigrated with purified chicken gizzard MLCK.

After optimization of the electroinjection conditions, we assessed the effects of electroinjecting MK-Ab on macrophage motility. MK-Ab are affinity-purified rabbit antibodies to turkey gizzard smooth muscle MLCK that have been characterized extensively (11-13). They are monospecific antibodies that exhibit wide cross-reactivity with MLCK found in smooth muscle and nonmuscle cells. On a Western blot analysis, MK-Ab bind to a macrophage protein with M, = 130,000 (Fig. 2). In addition, these antibodies inhibit the catalytic activity of smooth muscle and non-muscle MLCK (11, 13), including macrophage MLCK (Fig. 2).

The effect of electroinjecting MK-Ab on macrophage motility is shown in Fig. 3. Macrophages electroinjected with control antibodies display a characteristic chemotactic response, including high dose inhibition of migration (15), that is nearly identical to the response of untreated cells. In contrast, the incorporation of MK-Ab resulted in decreased migration at all concentrations of chemoattractant, and caused a 44 ± 6% decrease in the number of cells migrating at the optimal concentration of the chemoattractant.

To determine whether the degree of inhibition was dependent on the amount of MK-Ab introduced into the cells, macrophages were loaded with different amounts of antibody by electroinjection at different field strengths. Field strength, rather than protein concentration, was varied in these experiments for two reasons. First, there is a direct relationship between field strength and incorporation (Fig. 1 B). Second, MK-Ab precipitate when the concentration exceeds 2.5 mg/ml. Chemotaxis assays, performed at the optimal concentration of chemoattractant, demonstrated that decreased numbers of cells loaded with C-Ab migrated as the field strength was increased above 700 V/cm (Fig. 4). This was expected from the data in Fig. 1 A. However, MK-Ab–loaded
macrophages exhibited additional inhibition of migration at each field strength (Fig. 4). That is, fewer macrophages loaded with MK-Ab migrated at each field strength as compared with cells electroinjected with C-Ab.

We next investigated the effect of increasing intracellular MLCK activity on cell motility by electroinjecting MK- into macrophages. MLCK is unique in that it only catalyzes the phosphorylation of MLC20 (2). This is in contrast to most protein kinases, which phosphorylate multiple substrates (18). MK-, produced by digesting purified chicken gizzard MLCK with trypsin (21), is an unregulated (i.e., Ca2+/calmodulin-independent) form of the enzyme that retains its specificity for MLC20. We verified this by performing in vitro phosphorylation assays on extracts prepared from electroinjected cells. These experiments demonstrated that MK- retains its catalytic activity after electroinjection and that it only phosphorylates MLC20 (2) (data not shown).

When electroinjected into cells (Fig. 5), the number of cells that migrated decreased as a function of the MK- concentration in the electroinjection buffer. Since protein incorporation is directly related to the protein concentration in the electroinjection buffer and incorporation into the cells (32), chemotaxis was then performed using the optimal concentration of endotoxin-activated rat serum as defined in Fig. 2. Migration of cells electroinjected with MK- are reported as the percent of control cell migration at each protein concentration. The migration of control cells (i.e., cells electroinjected with trypsinized BSA) was virtually identical to the migration of cells incubated, but not electroinjected, with BSA. Each data point represents one experiment.

Finally, we investigated the effects of electroinjecting macrophages with MK-Ab or MK- into MLC20 phosphorylation and the distribution of myosin II (Table I). MLC20 phosphorylation was determined by immunoprecipitating myosin II from electroinjected cells. Cells electroinjected with C-Ab or MK-Ab were treated with a phosphoprotein phosphatase inhibitor, okadaic acid, that has been shown to increase MLC20 phosphorylation in macrophages (33). It was necessary to treat these cells with okadaic acid, because the level of MLC20 phosphorylation in untreated cells was so low (see lane marked BSA in Fig. 6) that the assay was not sensitive enough to demonstrate a decrease following electroinjection of MK-Ab. Treatings the cells with 1 μM okadaic acid raised the level of MLC20 phosphorylation sufficiently to permit detection of the effect of MK-Ab on MLC20 phosphorylation. Cells electroinjected with MK- or BSA did not require such manipulation. The data in Fig. 6 clearly demonstrate a decrease in MLC20 phosphorylation in cells electroinjected with MK-Ab compared to cells electroinjected with C-Ab, albeit under somewhat different conditions than used in Figs. 3 and 4 and in Table I. They also demonstrate an increase in MLC20 phosphorylation in cells in-
purified antibodies that have been shown to be monospecific and demonstrated relatively uniform labeling of all cells. Protein may not be in the cytoplasm (32). Finally, flow cytometry of cells electroporated with fluoresceinated BSA demonstrated relatively uniform labeling of all cells.

Both experimental probes that were electroporated into macrophages are extremely specific. MK-Ab and C-Ab are affinity-purified antibodies that have been shown to be monospecific for MLCK (Fig. 2 and refs. 11-13), whereas MK-Ab maintains the specificity of the parent enzyme for MLC20 (21).

We have investigated the importance of MLC20 phosphorylation in mediating cell motility by using antibodies to MLCK and a constitutively active form of MLCK to vary intracellular MLCK activity. These proteins were electroporated into living cells. Electroporation is a modification of the voltage discharge technique that has previously been used for transfecting (34), permanently permeabilizing, or fusing cells (35). Electroporation differs from these earlier applications in that it uses milder conditions and is designed to maintain high levels of cell viability. Since electroporation has not been used extensively, we performed a number of experiments to characterize the effect of subjecting macrophages to electrical discharges. Data from these experiments demonstrate that viability, as judged by trypan blue exclusion and by a physiological assay (Fig. 1), remains high when electroporation is performed under carefully defined conditions. It is also possible to electroporate large amounts of protein into cells. Based on an average diameter of 16 μm, the calculated concentration of rhodamine-labeled BSA in cells shocked at 700 V/cm, 15 μF is 2 μM. However, all of this protein may not be in the cytoplasm (32). Finally, flow cytometry of cells electroporated with fluoresceinated BSA demonstrated relatively uniform labeling of all cells.

| Table I. Relative Change in MLC20 Phosphorylation |
|-----------------------------------------------|
| MK-Ab                                       |
| 0.68 ± 0.15                                 |
| MK                                          |
| 1.77 ± 0.23                                 |

Changes in MLC20 phosphorylation in macrophages electroinjected with MK-Ab or MK- were compared to their respective controls (n = 3). Relative MLC20 phosphorylation was quantitated as described in Materials and Methods. Note that cells electroinjected with MK-Ab or C-Ab were treated with 1 μM okadaic acid, whereas cells electroporated with MK- or trypsin-treated BSA were not treated with okadaic acid.

The distribution of myosin II in electroporated cells was also quantitated because MLC20 phosphorylation affects filament formation by myosin II (10, 27). Electroporated cells were extracted with Triton and the myosin II in the Triton-soluble (cytoplasmic) and Triton-insoluble (cytoskeletal) fractions was quantitated by Western blotting, using antibodies to platelet myosin II, in order to identify unequivocally the myosin II heavy chains in each fraction and to increase the sensitivity of the assay. As shown in Table II, electroinjection of MK-Ab did not affect the distribution of myosin II. In contrast, there is almost a twofold increase in the amount of myosin II associated with the cytoskeleton in cells electroporated with MK- compared to cells electroinjected with BSA, C-Ab, or MK-Ab.

**Discussion**

We have investigated the importance of MLC20 phosphorylation in mediating cell motility by using antibodies to MLCK and a constitutively active form of MLCK to vary intracellular MLCK activity. These proteins were electroporated into living cells. Electroporation is a modification of the voltage discharge technique that has previously been used for transfecting (34), permanently permeabilizing, or fusing cells (35). Electroporation differs from these earlier applications in that it uses milder conditions and is designed to maintain high levels of cell viability. Since electroporation has not been used extensively, we performed a number of experiments to characterize the effect of subjecting macrophages to electrical discharges. Data from these experiments demonstrate that viability, as judged by trypan blue exclusion and by a physiological assay (Fig. 1), remains high when electroporation is performed under carefully defined conditions. It is also possible to electroporate large amounts of protein into cells. Based on an average diameter of 16 μm, the calculated concentration of rhodamine-labeled BSA in cells shocked at 700 V/cm, 15 μF is 2 μM. However, all of this protein may not be in the cytoplasm (32). Finally, flow cytometry of cells electroporated with fluoresceinated BSA demonstrated relatively uniform labeling of all cells.

Both experimental probes that were electroporated into macrophages are extremely specific. MK-Ab and C-Ab are affinity-purified antibodies that have been shown to be monospecific for MLCK (Fig. 2 and refs. 11-13), whereas MK-Ab maintains the specificity of the parent enzyme for MLC20 (21). Data from experiments using these proteins provide compelling evidence in support of the hypothesis that MLCK activity and MLC20 phosphorylation are important elements in mediating mammalian cell motility. Two major conclusions can be drawn from these results. First, changes in MLCK activity inhibit cell motility. Second, the data indicate that MLC20 phosphorylation must be maintained within narrow limits to support motility. Apparently, an increase or a decrease in MLC20 phosphorylation is sufficient to inhibit motility.

The explanation for these observations is complex. Since MK-Ab decreases MLC20 phosphorylation and MK- increases it (Fig. 6 and Table I), these proteins probably inhibit motility by affecting ATP hydrolysis and myosin II polymerization/depolymerization in different ways. Cell locomotion requires the simultaneous extension of the leading edge and retraction of the trailing edge, processes that are thought to depend on myosin I (17) and/or actin polymerization (6, 29) and a MLCK-catalyzed, myosin II-mediated contractile event (6, 8, 17, 20, 26), respectively. This, in turn, suggests that motile cells require exquisitely sensitive regulatory mechanisms to determine the spatial distribution of these reactions. MK-Ab appears to prevent motility by inhibiting ATP hydrolysis by actin and myosin II, thereby preventing tail retraction, whereas MK- may inhibit motility by stimulating ATP hydrolysis in spatially inappropriate regions of the cell. Thus, MK-Ab and MK- may affect motility by disrupting the spatial and temporal integration of ATP hydrolysis by actin and myosin II in opposite ways.

Simultaneously, changes in MLC20 phosphorylation could also have a profound effect on myosin filaments and the cytoskeleton. Motile cells continually change shape as they migrate from one point to another and the continual reorganization of the cytoskeleton is essential for cell motility (5, 28). It is now well-established that MLC20 phosphorylation regulates both ATP hydrolysis (2, 30, 31) and the ability of mammalian smooth muscle and nonmuscle myosin II to form filaments (10, 27). Unphosphorylated myosin II resides in a folded, 10S conformation, while phosphorylated myosin II has a sedimentation coefficient of 6S and an extended conformation. Most importantly, only phosphorylated 6S myosin II can form filaments (10, 27).

Therefore, we investigated the effect of MK-Ab and MK-
on the intracellular distribution of myosin II. MK-Ab reduced MLC20 phosphorylation (Fig. 6 and Table I) but had no effect on myosin II distribution (Table II). MK-, meanwhile, increased both MLC20 phosphorylation (Fig. 6 and Table I) and the amount of myosin II associated with the cytoskeleton (Table II). These data suggest that MK-Abs inhibit motility by inhibiting MLCK activity and decreasing MLC20 phosphorylation rather than preventing the polymerization of myosin II into filaments. In contrast, the introduction of MK- appears to result in an increase in myosin II filaments, secondary to an increase in MLC20 phosphorylation. Consequently, MK- appears to inhibit motility by stimulating ATP hydrolysis in spatially inappropriate locations and/or by decreasing the fluidity of the cytoskeleton by stimulating the polymerization of myosin II into filaments.

What, then, can be said about the relative importance of myosin I and myosin II in mammalian cell motility? Although myosin I has not been identified in macrophages, we cannot exclude the possible existence of myosin I in these cells. However, a myosin I light chain kinase has not been described in vertebrate tissues and turkey gizzard MLCK does not phosphorylate myosin I purified from the chicken brush border (J. Collins, Eastern Virginia Medical School, personal communication). These data and the specificity of MKAbs suggest that they are affecting myosin I should it exist in macrophages. Consequently, our data establish a central role for myosin II in mammalian cell motility by demonstrating that an increase or a decrease in MLCK activity and in MLC20 phosphorylation adversely affect macrophase motility.

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