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

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Abstract. Myosin II purified from mammalian nonmuscle cells is phosphorylated on the 20-kD light chain subunit (MLC_{20}) by the Ca^{2+}/calmodulin-dependent enzyme myosin light chain kinase (MLCK). The importance of MLC_{20} phosphorylation in regulating cell motility was investigated by introducing either antibodies to MLCK (MK-Ab) or a Ca^{2+}/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 MLC_{20} phosphorylation while MK^- increased MLC_{20} 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 MLC_{20} 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 (MLC_{20})[1] by myosin light chain kinase (MLCK) (2). Therefore, MLCK and MLC_{20} 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 *Dictyostelium discoideum* have questioned the importance of filamentous myosin in cell motility (14, 22). *Dictyostelium* 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 *Dictyostelium ameba* (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 *Dictyostelium* myosin II are regulated by both heavy chain and light chain phosphorylation (24). Interestingly, *Dictyostelium* 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-Abs) 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 MLC_{20} 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 translation 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

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1. *Abbreviations used in this paper*: C-Ab, control antibody; MK^-, trypsinized, constitutively active, myosin light chain kinase; MK-Ab, affinity-purified antibodies to myosin light chain kinase; MLCK, myosin light chain kinase; MLC_{20}, the 20-kD light chain of myosin.

decreasing MLCK activity on MLC_{20} phosphorylation and macrophage chemotaxis were then determined.

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^- was prepared by digesting purified chicken gizzard MLCK with trypsin as previously described (21). BSA was also digested with trypsin at a similar protein/trypsin ratio (40:1), and used as a control in the MK^- experiments. Trypsin was inactivated by adding a 100-fold excess of soybean trypsin inhibitor to the BSA and MLCK digests.

Cells

Alveolar macrophages were collected by lung lavage of healthy male 250–275 g Sprague-Dawley rats. Macrophages were centrifuged at 400 *g* for 5 min and 2.5×10^6 cells were resuspended in 0.4 ml electroinjection buffer (140 mM NaCl, 25 mM Hepes, 0.75 mM Na_2HPO_4, pH 7.15) containing the protein to be electroinjected.

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 recollected by centrifugation and resuspended in Gey's balanced salt solution (Grand Island Biological Company, Grand Island, NY) containing 1% BSA at $\sim 2 \times 10^6$ cells/ml. In all experiments, viability was assessed after electroinjection 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 (15). 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 (19). The macrophages migrated toward the chemoattractant passing through a polyvinylpyrrolidone-coated 5-μm pore size filter. After a 90-min incubation at 37°C, the unmigrated 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 2×10^6 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, 1.0 mM EDTA, 10 mM DTT, 10 mM pyrophosphate, 25 mM Tris, pH 7.5, 2 mg/l leupeptin and pepstatin, 100 mg/l soybean trypsin inhibitor, 180 mg/l phenylmethylsulfonylfluoride, 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 $(NH_4)_2SO_4$. 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 DTT, 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 $CaCl_2$, 0.1 μM calmodulin, 10 mM $MgCl_2$, 10 μM purified chicken gizzard MLC_{20}, 100 μM γ-^{32}P ATP 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 MLC_{20}, was subtracted from the phosphorylation data.

Western Blot Analysis

Macrophage (10^6 cells) and chick embryo fibroblasts (10^6 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 and 4°C for 15 h in 20% methanol, 25 mM Tris-192 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 M_r = 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

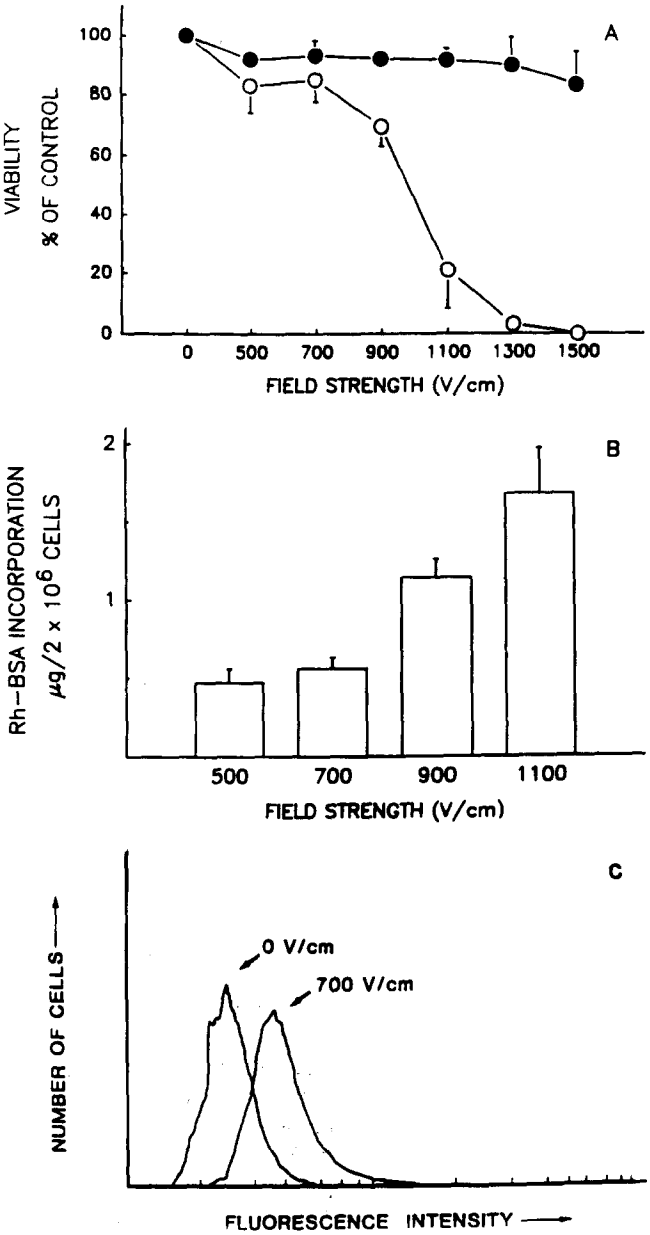

Figure 1. Characterization of the electroinjection procedure. (*A*) The relationship between field strength and viability as determined by trypan blue exclusion (●) and the chemotaxis assay (○) (*B*) The relationship between field strength and protein incorporation. (*C*) Analysis of electroinjected cells using fluorescence-activated cell sorting. (*A*) Macrophages electroinjected with 2.5 mg/ml C-Ab at different field strengths were recollected by centrifugation and resuspended in Gey's balanced salt solution containing 1% BSA at ~2 × 10^6 cells/ml. Trypan blue (0.4%) was added to an aliquot of cells and viability was quantified by determining the percentage of cells that excluded dye. Directed chemotaxis was performed on a separate aliquot of cells as described in Materials and Methods. The data points are the mean ± SE for three separate experiments done in triplicate in each category and they represent percent migration based on nonelectroinjected cells (0 V/cm) as 100%. (*B*) Macrophages were electroinjected at different field strengths in the presence of 2.5 mg/ml rhodamine-labeled BSA (*Rh-BSA*). Cells were washed three times in Gey's solution before quantitating the fluorescence as described in Materials and Methods. The figure depicts the mean ± SE of seven separate experiments. Increased incorporation of BSA with increasing field strength was significant ($p \leq 0.001$) as determined by a one-way analysis of variance. (*C*) Rat alveolar macrophages either incubated or electroinjected in the presence of 2.5 mg/ml fluorescein-labeled BSA were analyzed by flow cytometry as described in Materials and Methods. The spectra illustrate the number of cells in each fluorescence channel that were either only incubated with (0 V/cm) or electroinjected at 700 V/cm with fluorescein-labeled BSA. Native macrophage fluorescence was subtracted from both spectra. The x-axis is a log scale and these spectra are representative of three separate experiments.

transfer and very little (<5%) of the heavy chain was detected on the blot from the second transfer. Transfer of MLC_{20} was investigated by separating two sets of molecular weight standards and 50, 100, and 200 ng of purified, ^{32}P-labeled MLC_{20} 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 MLC_{20} 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.

MLC_{20} Phosphorylation

Macrophages (3 × 10^6 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 μF. The cells were collected, resuspended, and incubated in 0.5 ml of phosphate-free Gey's buffer containing 0.5 mCi of ^{32}P 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 MLC_{20} in each lane was excised and the radioactivity quantitated by liquid scintillation counting. The MLC_{20} 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 MLC_{20} 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. 1 *A* 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. 1 *B* 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. 1 *C*). These experiments demonstrated a single population of labeled cells that contained more fluorescence than cells merely incubated with the FITC-BSA. Since

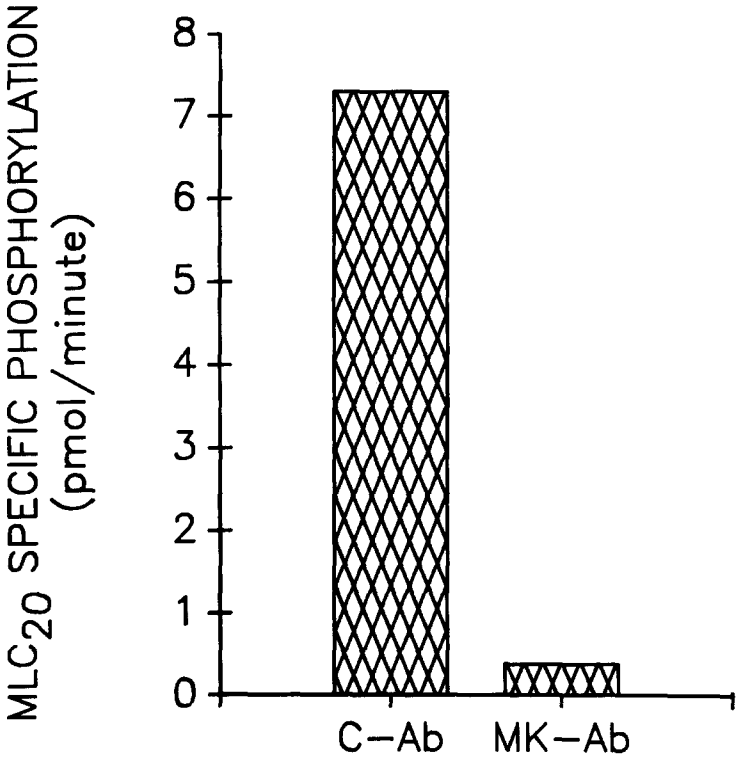

Figure 2. Interaction of MK-Ab with macrophage MLCK. (*left panel*) Macrophage MLCK was concentrated by ammonium sulfate fractionation (Materials and Methods). The effect of preincubating aliquots of this MLCK fraction with C-Ab or MK-Ab on MLC_{20} phosphorylation was then determined as described in Materials and Methods. The data demonstrate inhibition of ^{32}P incorporation by MK-Ab. (*right panel*) Western blot analysis of macrophage (*M*) and chick embryo fibroblasts (*CF*) proteins. Approximately 100 μg of macrophage and fibroblasts protein 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_r = 130,000 and comigrated with purified chicken gizzard MLCK.

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).

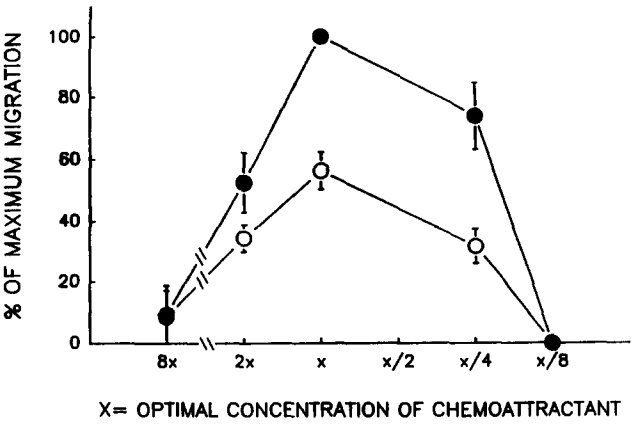

Figure 3. Effect of MK-Ab (○) and C-Ab (●) on macrophage motility as a function of chemoattractant concentration. Macrophages were electroinjected at 700 V/cm in the presence of 2.5 mg/ml of C-Ab or MK-Ab. The chemotaxis assay was performed using varying concentrations of endotoxin-activated rat serum. Percentage migration was calculated based on the number of cells electroinjected with C-Ab that migrated at the optimum concentration of endotoxin-activated rat serum as 100%. There was no statistically significant difference in the migration of untreated cells and cells electroinjected with C-Ab. However, inhibition of migration due to MK-Ab incorporation was significantly decreased ($p < 0.001$) as determined by analysis of variance (9) (n = 3).

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-Abs bind to a macrophage protein with M_r = 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-Abs 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

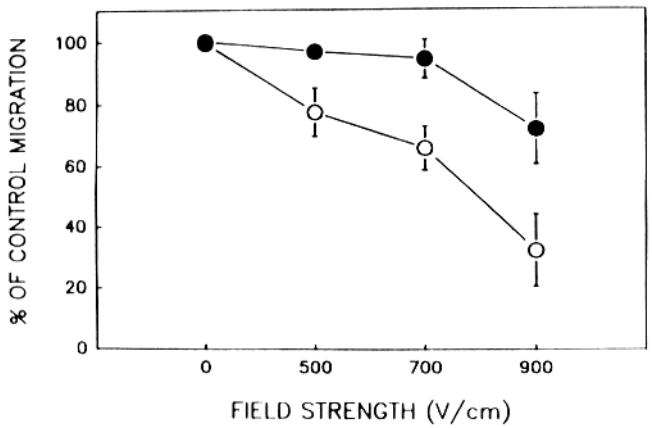

Figure 4. Effect of MK-Ab (○) and C-Ab (●) on macrophage motility as a function of increasing antibody incorporation. Macrophages were electroinjected at varying field strengths in the presence of 2.5 mg/ml of C-Ab or MK-Ab. Chemotaxis was performed at the optimal concentration of endotoxin-activated rat serum as defined in Fig. 2. Percent migration was calculated based on the number of cells incubated with C-Ab but not electroinjected (0 V/cm) that migrated as 100%. The data represent the means of two experiments at 500 and 900 V/cm. The data at 0 and at 700 V/cm ($n = 5$) include the mean values of the cells migrating at the optimal concentration of endotoxin-activated rat serum from the three experiments in Fig. 3. Data from C-Ab- and MK-Ab-treated cells were significant individually with p values < 0.001 when comparing field strength effects and MK-Ab effects, separately, on migration as determined by analysis of variance (9).

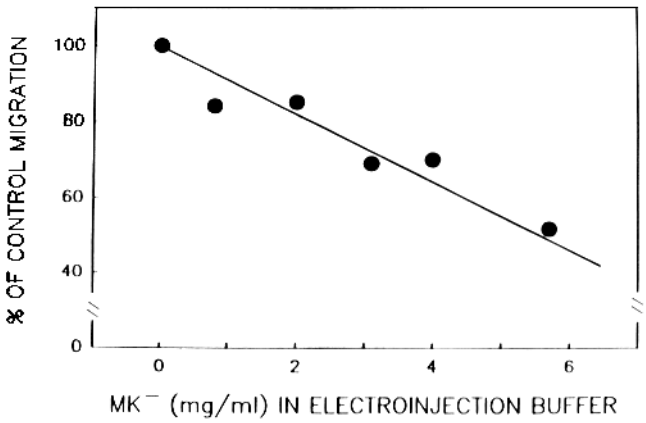

Figure 5. Effect of MK^- incorporation on macrophage motility. Macrophages were electroinjected at 700 V/cm in the presence of equivalent concentrations of trypsin-treated BSA (control cells) or MK^-. The protein concentration in the electroinjection buffer was varied as shown because there is a direct relationship between 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.

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 MLC_{20} (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., Ca^{2+}/calmodulin-independent) form of the enzyme that retains its specificity for MLC_{20}. 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 MLC_{20} (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 (32), these data demonstrate an inverse relationship between intracellular MLCK activity and cell motility.

Finally, we investigated the effects of electroinjecting macrophages with MK-Ab or MK^- on MLC_{20} phosphorylation and the distribution of myosin II (Table I). MLC_{20} 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 MLC_{20} phosphorylation in macrophages (33). It was necessary to treat these cells with okadaic acid, because the level of MLC_{20} 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. Treating the cells with 1 μM okadaic acid raised the level of MLC_{20} phosphorylation sufficiently to permit detection of the effect of MK-Ab on MLC_{20} phosphorylation. Cells electroinjected with MK^- or BSA did not require such manipulation. The data in Fig. 6 clearly demonstrate a decrease in MLC_{20} 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 II. They also demonstrate an increase in MLC_{20} phosphorylation in cells in-

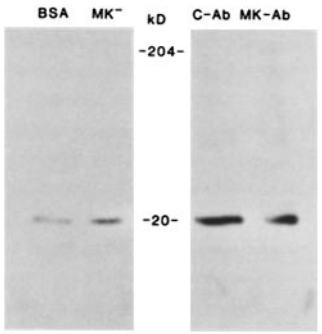

Figure 6. MLC_{20} phosphorylation in electroinjected macrophages. Macrophages (3×10^6 cells) were electroinjected with MK^-, trypsin-treated BSA, MK-Ab, or C-Ab at concentrations of 2.5 mg/ml at 700 V/cm. Myosin II was immunoprecipitated from cells incubated with ^{32}P as described in Materials and Methods. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and exposed to an X-ray film. A photograph of the X-ray film from a representative experiment out of three that were performed is shown in this figure. The numbers 204 and 20 refer to the molecular weights ($\times 10^{-3}$) of myosin II heavy chain and MLC_{20}.

Table I. Relative Change in MLC_{20} Phosphorylation

	MLC_{20} Phosphorylation (Fold change compared to control)
MK-Ab	0.68 ± 0.15
MK^-	1.77 ± 0.23

Changes in MLC_{20} phosphorylation in macrophages electroinjected with MK-Ab or MK^- were compared to their respective controls (n = 3). Relative MLC_{20} 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 electroinjected with MK^- or trypsin-treated BSA were not treated with okadaic acid.

jected with MK^- compared to cells electroinjected with trypsinized BSA. The changes in MLC_{20} phosphorylation were more quantitatively compared (Table I) by normalizing the data as described in Materials and Methods. The normalized data demonstrate that MLC_{20} phosphorylation is decreased in cells injected with MK-Ab and increased in cells injected with MK^- compared to their respective controls (Table I).

The distribution of myosin II in electroinjected cells was also quantitated because MLC_{20} phosphorylation affects filament formation by myosin II (10, 27). Electroinjected 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 electroinjected with MK^- compared to cells electroinjected with BSA, C-Ab, or MK-Ab.

Table II. Cellular Distribution of Myosin in Macrophages following Electroinjection of Various Proteins

Electroinjected protein	Myosin distribution % Cytoplasmic (Triton soluble)	% Cytoskeletal (Triton insoluble)
C-Ab (4)	72.3 ± 4.9	27.7 ± 4.9
MK-Ab (4)	69.6 ± 4.8	30.3 ± 4.8
BSA (3)	71.6 ± 5.1	28.4 ± 5.1
MK^- (3)	45.5 ± 9.7	54.5 ± 9.7

Macrophages were electroinjected at 700 V/cm, 15 μF in the presence of 2.5 mg/ml control antibodies (C-Ab), 2.5 mg/ml antibodies to MLCK (MK-Ab), 5.8 mg/ml trypsinized BSA or 5.8 mg/ml unregulated MLCK (MK^-). Myosin distribution was determined by treating cells with Triton X-100 as described in Materials and Methods. The number of trials in each experimental group is shown in brackets and the data represent the mean ± SE.

Discussion

We have investigated the importance of MLC_{20} 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 electroinjected into living cells. Electroinjection is a modification of the voltage discharge technique that has previously been used for transfecting (34), permanently permeabilizing, or fusing cells (35). Electroinjection differs from these earlier applications in that it uses milder conditions and is designed to maintain high levels of cell viability. Since electroinjection 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 electroinjection is performed under carefully defined conditions. It is also possible to electroinject 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 electroinjected with fluoresceinated BSA demonstrated relatively uniform labeling of all cells.

Both experimental probes that were electroinjected into macrophages are extremely specific. MK-Abs are affinity-purified antibodies that have been shown to be monospecific for MLCK (Fig. 2 and refs. 11-13), whereas MK^- maintains the specificity of the parent enzyme for MLC_{20} (21). Data from experiments using these proteins provide compelling evidence in support of the hypothesis that MLCK activity and MLC_{20} 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 MLC_{20} phosphorylation must be maintained within narrow limits to support motility. Apparently, an increase or a decrease in MLC_{20} phosphorylation is sufficient to inhibit motility.

The explanation for these observations is complex. Since MK-Abs decreases MLC_{20} 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-Abs appear 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 MLC_{20} 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 MLC_{20} 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 MLC_{20} phosphorylation (Fig. 6 and Table I) but had no effect on myosin II distribution (Table II). MK^-, meanwhile, increased both MLC_{20} 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 MLC_{20} 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 MLC_{20} 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 MK-Ab (11–13) and MK^- (21) argue against the possibility 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 MLC_{20} phosphorylation adversely affect macrophage motility.

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References

1. Adams, R. J., and T. D. Pollard. 1989. Binding of myosin I to membrane lipids. *Nature (Lond.).* 340:565–568.
2. Adelstein, R. S. 1983. Regulation of contractile proteins by phosphorylation. *J. Clin. Invest.* 72:1863–1866.
3. Adelstein, R. S., and M. A. Conti. 1975. Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity. *Nature (Lond.).* 256:597–598.
4. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1989. The Molecular Biology of the Cell, second edition, Garland Publishing, Inc., New York. 641–945.
5. Bray, D., and J. G. White. 1988. Cortical flow in animal cells. *Science (Wash. DC).* 239:883–887.
6. Cande, W. Z., and R. M. Ezzell. 1986. Evidence for regulation of lamellipodial and tail contraction of glycerinated chicken embryonic fibroblasts by myosin light chain kinase. *Cell Motil. Cytoskeleton.* 6:640–648.
7. Chakrabarti, R., D. E. Wylie, and S. M. Schuster. 1989. Transfer of monoclonal antibodies into mammalian cells by electroporation. *J. Biol. Chem.* 264:15494–15500.
8. Chen, W. T. 1981. Mechanism of retraction of the trailing edge during fibroblast movement. *J. Cell Biol.* 90:187–200.
9. Cody, R. P., and J. K. Smith. 1987. Hypothesis testing (two groups). *In* Applied Statistics and the SAS Programming Language. 2nd edition. R. P. Cody and J. K. Smith, editors. North Holland, NY. 92–95.
10. Craig, R., R. Smith, and J. Kendrick-Jones. 1983. Light-chain phosphorylation controls the conformation of vertebrate non-muscle and smooth muscle myosin molecules. *Nature (Lond.).* 302:436–439.
11. de Lanerolle, P., R. S. Adelstein, J. R. Feramisco, and K. Burridge. 1981. Characterization of antibodies to smooth muscle myosin kinase and their use in localizing myosin kinase in nonmuscle cells. *Proc. Natl. Acad. Sci. USA.* 78:4738–4742.
12. de Lanerolle, P., M. Nishikawa, D. A. Yost, and R. S. Adelstein. 1984. Increased phosphorylation of myosin light chain kinase after an increase in cyclic AMP in intact smooth muscle. *Science (Wash. DC).* 223:1415–1417.
13. de Lanerolle, P., M. Nishikawa, R. Felsen, and R. S. Adelstein. 1987. Immunological properties of myosin light-chain kinases. *Biochim. Biophys. Acta.* 914:74–82.
14. De Lozanne, A., and J. A. Spudich. 1987. Disruption of the Dictyostelium myosin heavy chain gene by homologous recombination. *Science (Wash. DC).* 236:1086–1091.
15. Falk, W., R. H. Goodwin, and E. J. Leonard. 1980. A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods.* 33:239–247.
16. Fox, J. E. B., and D. R. Phillips. 1982. Role of phosphorylation in mediating the association of myosin with the cytoskeletal structures of human platelets. *J. Biol. Chem.* 257:4120–4126.
17. Fukui, Y., T. J. Lynch, H. Brzeska, and E. D. Korn. 1989. Myosin I is located at the leading edges of locomoting Dictyostelium amoebae. *Nature (Lond.).* 341:328–331.
18. Greengard, P. 1978. Phosphorylated proteins as physiological effectors. *Science (Wash. DC).* 199:146–152.
19. Hausman, M. S., R. Snyderman, and S. E. Mergenhagen. 1972. Humoral mediators of chemotaxis of mononuclear leukocytes. *J. Infect. Dis.* 125:595–602.
20. Holzapfel, G., J. Wehland, and K. Weber. 1983. Calcium control of actin-myosin based contraction in triton models of mouse 3T3 fibroblasts is mediated by the myosin light chain kinase (MLCK)-calmodulin complex. *Exp. Cell Res.* 148:117–126.
21. Ikebe, M., M. Stepinska, B. E. Kemp, A. R. Means, and D. J. Hartshorne. 1987. Proteolysis of smooth muscle myosin light chain kinase. *J. Biol. Chem.* 260:13828–13834.
22. Knecht, D. A., and W. F. Loomis. 1987. Antisense RNA inactivation of myosin heavy chain gene expression in Dictyostelium discoideum. *Science (Wash. DC).* 236:1081–1085.
23. Korn, E. D. 1978. Biochemistry of actomyosin-dependent cell motility. *Proc. Natl. Acad. Sci. USA.* 75:588–599.
24. Korn, E. D., and J. A. Hammer. 1988. Myosins of nonmuscle cells. *Annu. Rev. Biophys. Biophys. Chem.* 17:23–45.
25. Ludowyke, R. I., I. Peleg, M. A. Beaven, and R. S. Adelstein. 1989. Antigen-induced secretion of histamine and the phosphorylation of myosin by protein kinase C in rat basophilic leukemia cells. *J. Biol. Chem.* 264:12492–12501.
26. Oster, G. F. 1984. On the crawling of cells. *J. Embryol. Exp. Morphol.* 83(Suppl):329–364.
27. Scholey, J. M., R. C. Smith, D. Drenckhahn, U. Groschel-Stewart, and J. Kendrick-Jones. 1982. Thymus myosin. Isolation and characterization of myosin from calf thymus and thymic lymphocytes, and studies on the effect of phosphorylation of its Mr = 20,000 light chain. *J. Biol. Chem.* 257:7737–7745.
28. Snyderman, R., and E. J. Goetzl. 1981. Molecular and cellular mechanisms of leukocyte chemotaxis. *Science (Wash. DC).* 213:830–837.
29. Tilney, L. G., S. Hatano, H. Ishikawa, and M. S. Mooseker. 1973. The polymerization of actin: its role in the generation of the acrosomal process of certain echinoderm sperm. *J. Cell Biol.* 59:109–126.
30. Trotter, J. A., and R. S. Adelstein. 1979. Macrophage myosin. Regulation of actin-activated ATPase activity by phosphorylation of the 20,000-dalton light chain. *J. Biol. Chem.* 254:8781–8785.
31. Wagner, P. D., N.-D. Vu, and J. N. George. 1985. Random phosphorylation of the two heads of thymus myosin and the independent stimulation of their actin-activated ATPases. *J. Biol. Chem.* 260:8084–8089.
32. Wilson, A. K., J. Horwitz, and P. de Lanerolle. 1991. Evaluation of the electroinjection method for introducing proteins into living cells. *Am. J. Physiol.* 260:C355–C363.
33. Wilson, A. K., A. Takai, J. C. Ruegg, and P. de Lanerolle. 1991. Okadaic acid, a phosphatase inhibitor, decreases macrophage motility. *Am. J. Physiol.* 260:L105–L112.
34. Wong, T.-K., and E. Neumann. 1982. Electric field mediated gene transfer. *Biochem. Biophys. Res. Commun.* 107:584–587.
35. Zimmerman, U. 1986. Electrical breakdown, electropermeabilization and electrofusion. *Rev. Physiol. Biochem. Pharmacol.* 105:175–256.