Regulation of polymorphonuclear leukocyte phagocytosis by myosin light chain kinase after activation of mitogen-activated protein kinase

Pamela J. Mansfield, James A. Shayman, and Laurence A. Boxer

Polymorphonuclear leukocyte (PMNL) phagocytosis mediated by FcγRII proceeds in concert with activation of the mitogen-activated protein (MAP) kinase, extracellular signal-regulated kinase (ERK). We hypothesized that myosin light chain kinase (MLCK) could be phosphorylated and activated by ERK, thereby linking the MAP kinase pathway to the activation of cytoskeletal components required for pseudopod formation. To explore this potential linkage, PMNLs were challenged with antibody-coated erythrocytes (EIgG). Peak MLCK activity, 3-fold increased over controls, occurred at 4 to 6 minutes, corresponding with the peak rate of target ingestion and ERK2 activity. The MLCK inhibitor ML-7 (10 μmol/L) inhibited both phagocytosis and MLCK activity to basal values, thereby providing further support for the linkage between the functional response and the requirement for MLCK activation. The MAP kinase (MEK) inhibitor PD98059 inhibited phagocytosis, MLCK activity, and ERK2 activity by 80% to 90%. To directly link ERK activation to MLCK activation, ERK2 was immunoprecipitated from PMNLs after EIgG ingestion. The isolated ERK2 was incubated with PMNL cytosol as a source of unactivated MLCK and with MLCK substrate; under these conditions ERK2 activated MLCK, resulting in phosphorylation of the MLCK substrate or of the myosin light chain itself. Because MLCK activates myosin, we evaluated the effect of directly inhibiting myosin adenosine triphosphatase using 2,3-butanedione monoxime (BDM) and found that phagocytosis was inhibited by more than 90% but MLCK activity remained unaffected. These results are consistent with the interpretation that MEK activates ERK, ERK2 then activates MLCK, and MLCK activates myosin. MLCK activation is a critical step in the cytoskeletal changes resulting in pseudopod formation.

Introduction

Polymorphonuclear leukocytes (PMNLs) are recruited by chemotactic tractive forces to sites of inflammation where they ingest, or phagocytose, opsonized particles. Two classes of PMNL receptors are involved in phagocytosis: the Fc receptor and the complement receptor. The sequence of signal transduction events from FcγRIIa receptor ligation leading to the ingestion of IgG-opsonized particles is partially known. One of the first steps after ligand binding to the Fc receptor is activation of several nonreceptor protein tyrosine kinases, such as Syk, p55γc, and p59fyn. Subsequently, phospholipase D is activated during PMNL phagocytosis and generates phosphatidic acid. Phosphatidate phosphohydrolase generates diglyceride from phosphatidic acid, thereby activating protein kinase C (PKC). The Ca++-dependent PKCδ appears to be important to PMNL phagocytosis. Our group has recently shown that PKCδ and Raf-1 translocate to the plasma membrane during the first few minutes of PMNL phagocytosis. In most systems, Raf-1 then initiates the mitogen-activated protein (MAP) kinase cascade. Previously we showed that activation of the MAP kinases ERK2 and ERK1, as well as phospholipase D, are required for the ingestion of IgG-opsonized particles. Others have also found ERK activity to increase during both FcR- and CR3-mediated phagocytosis and the oxidative burst. The in vivo substrate for ERK2 during PMNL phagocytosis has not been determined.

The later part of the phagocytic signaling pathway involves activation of the cytoskeletal components actin and myosin. Actin polymerization is required for pseudopod extension and particle engulfment, whereas myosin furnishes propulsive force by its interaction with actin. Actin polymerization is initiated by many stimuli, including cross-linking of FcγRIIa. Myosin concentrates in the anterior pseudopod of phagocytosing PMNLs, where it binds F-actin and hydrolyzes adenosine triphosphate (ATP). Myosin converts ATP hydrolysis energy to movement through conformational change of the myosin molecule while it is bound to actin. Nonmuscle myosin II ATPase activity is up-regulated by light chain phosphorylation, relieving the inhibitory effect of the light chains, and this phosphorylation is accomplished by myosin light chain kinase (MLCK). PMNL chemotaxis and random migration involve MLCK activation and myosin phosphorylation. Klemke et al. used COS-7 cells transfected with constitutively active MEK to show that MLCK is phosphorylated and activated by the MAP kinase ERK2, and these signals are required for chemotaxis. Because the MAP kinase cascade and cytoskeletal interactions are known to be critical to PMNL phagocytosis, we conjectured that these were
similarly linked in phagocytic signaling. In this study, we demonstrate that ERK2, activated by MEK, activates MLCK during PMNL phagocytosis and that this is followed by the activation of myosin ATPase.

Materials and methods

Reagents

Peptide substrate specific for myosin light chain kinase, corresponding to residues 11-23 of smooth muscle myosin light chain, was obtained from Biolom (Plymouth Meeting, PA). The myosin light chain kinase inhibitor ML-7, (5-iodonaphthalene-1-sulfonyl) homopiperazine, was purchased from Calbiochem (San Diego, CA). 2,3-Butanediene 2-monoxime (BDM) was obtained from Sigma (St. Louis, MO). The MEK inhibitor PD098059 (2′-amino-3′-methoxyflavone) was a gift from Alan R. Saltiel, Parke-Davis (Ann Arbor, MI). Polyclonal antibody against ERK2 (p42; C-14) and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-ERK recognizing both p42 and p44 was a gift from Dr. Robert Adelstein’s laboratory (National Institutes of Health, Bethesda, MD).

Cells

PMNLs were isolated from peripheral venous blood from healthy volunteers as previously described.15 PMNLs were pretreated with 5 mmol/L diisopropyl fluorophosphate on ice for 5 minutes, then washed 3 times with phosphate-buffered saline (PBS). Incubation with inhibitors was performed before phagocytosis as follows: PMNLs were incubated with the indicated concentrations of ML-7 or BDM for 10 minutes at 37°C or with 50 µmol/L PD098059 for 30 minutes at 22°C.

Phagocytosis and preparation of PMNL lysates

Phagocytosis was conducted as previously described.7,28 Briefly, sheep erythrocytes (E) were opsonized with anti-sheep E antibody (EIGG) (Cappel Organon Teknika, Durham, NC). PMNLs (×10^6/mL) were warmed at 37°C for 3 minutes, and EIGG was added to PMNLs at a ratio of 50:1. No priming agent was used. Samples were removed at the indicated time points and microfuged for 7 seconds, then nonlignest EIGG were lysed with double-distilled water containing 1 mmol/L Na3VO4 and 50 mmol/L NaF. PMNLs were returned to isotonicity by adding KCl, microfuged, and suspended in MLCK kinase buffer (40 mmol/L HEPES pH 7.0, 5 mmol/L Mg acetate, 0.55 mmol/L CaCl2, and 0.1% Tween-80) containing freshly added 1 mmol/L Na3VO4, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL pepstatin, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 100 µg/mL soybean trypsin inhibitor. Samples were probe sonicated on ice for 12 seconds 2 times and microfuged, and suspended in scintillation fluid and counted in a scintillation counter.

Protein substrate specific for myosin light chain kinase, corresponding to residues 17-23 of smooth muscle myosin light chain, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-ERK recognizing both p42 and p44 was a gift from Dr. Robert Adelstein’s laboratory (National Institutes of Health, Bethesda, MD).

Kinase assay

Each PMNL lysate sample was divided into 2 equal aliquots, 1 to receive substrate and 1 to substrate. A saturating concentration of substrate, 300 µmol/L, was chosen for this assay. A reaction mixture containing substrate peptide or buffer, 5 µCi [γ-32P]ATP, and 0.5 mmol/L unlabeled ATP was added to each tube, and samples were incubated for 10 minutes at 30°C. The reaction was terminated by filtering through Whatman P81 paper (Clifton, NJ). Filters were added to scintillation fluid and placed in a scintillation counter (Wallac, Gaithersburg, MD). Blanks were assay samples run without substrate. One-sample, 2-tailed Student t tests were used to assess statistical significance of increases in kinase activity.

Orthophosphate labeling and immunoprecipitation of myosin

PMNLs were suspended at 10^6/mL in 30 mmol/L HEPES pH 7.4, 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, 10 mmol/L glucose, 2 mmol/L bovine serum albumin, and 1 mol/L H3[32P]O4. They were incubated at 37°C for 30 minutes, then washed with PBS and suspended in PBS with 1 mmol/L CaCl2 and 1 mmol/L MgCl2. PMNLs were allowed to phagocytose EIGG for 4 minutes, then samples were lysed with buffer containing 1% NP-40, 250 mmol/L NaCl, 5 mmol/L EGTA, 20 mmol/L Tris pH 8, 40 mmol/L 4-nitrophenyl phosphate, and phosphatase inhibitors and protease inhibitors as described above. Lysates were incubated overnight with antiplatelet myosin, then for 2 hours with Protein A-Sepharose. Samples were subjected to 12% SDS-PAGE, and the gels were dried and exposed to X-ray film.

Preparation of cytosol

PMNLs were isolated and DFP-treated as described above. They were suspended at 10^6/mL in extraction buffer (50 mmol/L Tris pH 7.5, 2 mmol/L EGTA, 1 mmol/L PMSF, 1 µg/mL leupeptin, 10 µmol/L benzamidinie, 10 µmol/L pepstatin, and 0.2 µg/mL aprotinin) and were probe sonicated for 12 seconds 2 times. Extracts were centrifuged for 10 minutes at 8000g, and the supernatants were layered on 15% sucrose in 10 mmol/L Tris, pH 7.5. The samples were centrifuged in a swinging bucket rotor at 150 000 × g for 30 minutes. Cytosol was removed from the top half of the upper layer and used within 2 hours.

ERK2 immunoprecipitation and coupled kinase assay

PMNLs were allowed to phagocytose for 5 minutes as described above; controls were incubated at 37°C for the same interval. PMNLs (×10^3) were lysed in 800 µL buffer containing 50 mmol/L HEPES (pH 7.5), 100 mmol/L NaCl, 2 mmol/L EDTA, 1% Nonidet P-40, 1 µmol/L pepstatin, 1 µg/mL leupeptin, 0.2 mmol/L PMSF, 0.5 mmol/L sodium orthovanadate, 50 mmol/L NaF, 2 µg/mL aprotinin, and 40 mmol/L 4-nitrophenyl phosphate. Precleared lysates were incubated with 1 µg anti-ERK2 overnight at 4°C while rotating. Protein A-Sepharose was added to each sample and incubated for 1 hour at 4°C while rotating. Beads were washed twice with cold lysis buffer, then twice with cold MLCK kinase buffer. Cytosol from resting PMNLs, or buffer, was added to samples as indicated (10 cell equivalents/sample). Beads were then resuspended in MLCK kinase buffer containing 500 mmol/L ATP, 5 µCi/sample [γ-32P]ATP, and 300 µmol/mL MLCK peptide substrate or buffer for blanks. Samples were incubated for 10 minutes at 30°C and microfuged for 30 seconds. The reaction was terminated by filtering supernatants through Whatman P81 paper. Filters were added to scintillation fluid and counted in a scintillation counter.

Counts were expressed as percentage of controls from 22°C PMNLs. Paired, 2-tailed Student t tests were used to assess differences between treatments.

These experiments were also conducted using a crude PMNL myosin, prepared according to Boxer and Stossel,30 as substrate. After treatment with ERK2, the myosin was immunoprecipitated as described in “Orthophosphate Labeling and Immunoprecipitation of Myosin.” Incorporation of [32P] was measured by scintillation counting of the immunoprecipitate.

Immunoblotting

To ensure equal ERK2 was immunoprecipitated from each sample, immunoblotting was performed on samples. Beads were combined with sample buffer,31 boiled 5 minutes, and run on 10% SDS-PAGE mini-gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Schleicher and Schuell, Keene, NH) and then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween-20. The membrane was probed with anti-ERK, which recognizes 42- and 44-kd MAP kinases diluted 1:1000 in blocking buffer, and washed 3 times with 0.2% Tween-20 in TBS. The membrane was then incubated with horseradish peroxidase-conjugated sheep antirabbit antibody (Santa Cruz), diluted 1:10 000 in blocking buffer, and again washed 3 times. Phosphorylated bands were visualized using enhanced chemiluminescence (ECL) detection reagents.
increased more than 3-fold during phagocytosis, in contrast to control PMNLs (maintained for up to 10 minutes without phagocytic targets) which showed no increase in activity (Figure 1A). Unopsonized erythrocytes were not ingested by PMNL and did not stimulate MLCK activity (data not shown). The MLCK activity peaked at 4 to 6 minutes, corresponding to ElgG ingestion, which also reaches a maximum rate within 5 minutes of initiating phagocytosis.3 Parallelling the phagocytic rate, MLCK activity decreased to baseline by 10 minutes. To demonstrate phosphorylation of myosin light chain, we labeled PMNLs with 32P and immunoprecipitated myosin from detergent lysates using antiplatelet myosin. We observed a single band at approximately 20 kD whose intensity was increased in samples from phagocytosing PMNLs compared with controls (Figure 1B). This band was most likely myosin light chain based on its relative mobility and its recognition by antiplatelet myosin. In prior studies we observed ERK2 activity to peak by 3 minutes,15 consistent with the hypothesis that ERK2 resides upstream of MLCK in the phagocytic signaling pathway.

Because the time course of MLCK activity corresponded to the rate of phagocytosis, we next determined whether the inhibition of MLCK would block phagocytosis. PMNLs were incubated for 10 minutes with ML-7, a MLCK inhibitor,36 before assaying for phagocytosis and MLCK activity. Phagocytosis was inhibited partially (50%) using 3 µmol/L ML-7 and almost completely at 10 µmol/L ML-7 (Figure 2). Similarly, MLCK activity was also inhibited to 13% of control rates in the presence of 10 µmol/L ML-7 (Figure 2). The observed inhibition of phagocytosis supports the notion that MLCK activation is linked to the functional response of particle ingestion. To test the specificity of ML-7 for phagocytosis and its link to MLCK, we conducted assays of fMLP-stimulated (100 nmol/L) intracellular Ca2+ transients and phagocytosis-stimulated tyrosine phosphorylation of Syk. Neither response was significantly blocked by ML-7 (Table 1). In addition, the binding of ElgG to PMNLs, a preliminary step for phagocytosis, was tested at 4°C (to preclude ingestion). ML-7 had no effect on ElgG binding to PMNLs (Table 1). These data are consistent with the selective inhibition of MLCK activity by ML-7.

Previously we and others have found that ERK2 and ERK1 are activated by MEK during PMNL phagocytosis and in other cell

**Results**

MLCK activity was measured using lysates from phagocytosing PMNL and peptide substrate specific for MLCK to determine the kinetics of MLCK activation. The peptide substrate was previously characterized to have an apparent Km of 7.5 µmol/L,34 comparable to the Km of 8.6 µmol/L for myosin light chain.33 MLCK activity
Table 1. Effect of inhibitors on selected PMNL functions other than phagocytosis

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<th>Syk phosphorylation</th>
<th>Intracellular Ca(^{2+}) transient</th>
<th>Binding of EIgG to PMNLs</th>
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<tr>
<td>Control</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
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<tr>
<td>ML-7</td>
<td>90.5 ± 30.5</td>
<td>87.0 ± 13.0</td>
<td>101.8 ± 3.0</td>
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<td>BDM</td>
<td>86.0 ± 14.0</td>
<td>88.9 ± 6.4</td>
<td>104.6 ± 2.7</td>
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PMNLs were incubated with 10 µmol/L ML-7 or 10 mmol/L BDM for 10 minutes at 37°C before stimulation. For Syk phosphorylation, PMNLs were challenged with EIgG for 3 minutes, lysed, and immunoblotted with anti-phosphotyrosine; blots were scanned with a densitometer. For measurement of Ca\(^{2+}\) transients, PMNLs were loaded with fluo-3 and stimulated with 100 nmol/L fMLP, and fluorescence was monitored continuously; peak and duration of fluorescence were evaluated. Binding of EIgG was evaluated after 30 minutes at 4°C by counting the number of PMNLs with bound erythrocytes; each PMNL bound many erythrocytes. Data are expressed as mean ± SEM.

After phosphorylation of myosin light chains by MLCK, both the association of myosin with actin and the actin-stimulated adenosine triphosphatase activity of myosin increase.\(^{23,24}\) To determine whether myosin activation is required for phagocytosis, we used BDM, a known inhibitor of nonmuscle myosin II and myosin V adenosine triphosphatases including those of platelets\(^{42}\) and macrophages.\(^{43}\) BDM has no effect on actin filaments in PtK2 cells\(^{42}\) or on the formation of actin-rich phagocytic cups in macrophages,\(^{43}\) implying it would not affect PMNL actin. PMNL phagocytosis was completely inhibited by 10 mmol/L BDM, whereas MLCK activity remained unaffected (Figure 4). This result indicated that the myosin ATPase activity lies downstream of MLCK and precluded nonspecific effects of BDM upstream of MLCK. To further evaluate the effects of BDM, we tested Ca\(^{2+}\), the phosphorylation of Syk, and the binding of EIgG to PMNL in the presence of BDM. None of these processes were significantly inhibited (Table 1), demonstrating the relative specificity of BDM. The inhibition of phagocytosis by BDM was consistent with the interpretation that myosin ATPase activity is required for phagocytosis, supplying the energy to extend the pseudopod and to permit ingestion of the particles.

Discussion

This study demonstrated that the activation of MLCK during PMNL phagocytosis is a result of ERK2 activation. Both the increase in phosphorylation of a specific MLCK substrate during phagocytosis and the concurrent inhibition of this phosphorylation and phagocytosis by an inhibitor of MLCK implicate MLCK activation as a requirement for the ingestion of IgG-opsonized particles. The ability of ERK2 immunoprecipitated from

![Graph showing the activation of MLCK by ERK2](image)

Figure 3. Activation of MLCK by ERK2. PMNLs were allowed to undergo phagocytosis of EIgG for 5 minutes or incubated without EIgG, then lysed with NP-40. ERK2 was immunoprecipitated from lysates to obtain activated and unactivated ERK2, respectively. Cytosol isolated from resting PMNLs or buffer was added to samples along with [γ-\(^{32}\)P]ATP and peptide substrate (plain bars), and kinase activity was quantitated as described for Figure 1, or PMNL myosin replaced peptide substrate (cross-hatched bars) and was immunoprecipitated. R, ERK2 immunoprecipitated from resting PMNLs incubated at 37°C; P, ERK2 immunoprecipitated from phagocytosing PMNLs; -, cytosol present; +, cytosol added. Data represent the mean ± SEM of 3 experiments. MLCK activity in PMNLs at 22°C = 100%. *Significantly different from control (left bar); P < .005. Other data are not significantly different from each other.
phagocytosing, but not resting, PMNLs to activate cytosolic MLCK demonstrated that ERK2 activation is necessary for MLCK activation. However, our data did not rule out the presence of a cytosolic intermediate between ERK2 and MLCK.

A proposed model of PMNL signaling during FcR-mediated phagocytosis is shown in Figure 5. We and others have investigated several aspects of this model. Our observations suggest that most of the signaling activity occurs in the first few minutes of phagocytosis, corresponding with the maximum rate of ingestion. Phospholipase D activity rises by 1 minute after initiation of ingestion and remains elevated for 30 minutes. Translocation of PKCβ and Raf-1 occurs at 3 minutes. ERK2 phosphorylation rises at 1 to 5 minutes and ERK2 activity peaks at 3 to 5 minutes. Syk phosphorylation similarly peaks at 3 to 5 minutes. Translocation of PKCβ and Raf-1 and the phosphorylation of ERK2 and Syk all decrease by 10 minutes into phagocytosis, a time at which the ingestion rate has steeply declined. Consistent with these observations, in the current study MLCK activity peaked at 4 to 6 minutes and decreased to baseline by 10 minutes. The temporal proximity of activation of these kinases suggested that they were closely linked signaling steps in the phagocytic signaling cascade.

Inhibition of phagocytosis, ERK2 activation, and MLCK activity by a specific MEK inhibitor demonstrates that these phenomena are regulated by MEK. Immunoprecipitated ERK2, which was activated during PMNL phagocytosis, could activate cytosolic MLCK, and this activity was inhibitable by ML-7. Our results implicate a pathway similar to that described for COS-7 cells by Klemke et al., who showed that MEK-dependent ERK2 activation led to the activation of MLCK to phosphorylate myosin light chain. Furthermore, Klemke et al demonstrated direct phosphorylation of MLCK by ERK2.

Some studies have suggested possible mediators of MLCK activity or myosin light chain phosphorylation other than ERK2. A cyclic adenosine monophosphate (cAMP)-dependent protein kinase phosphorylates and inactivates MLCK in fibroblasts. However, results from our laboratory suggest that cAMP has no significant role in PMNL phagocytosis of EfgG (data not shown). PI 3-kinase is activated during phagocytosis. Recent work in our laboratory suggests that PI 3-kinase activation, though essential for PMNL phagocytosis, acts downstream of the MAP kinase cascade, making it a possible activator of MLCK. However, in macrophages PI 3-kinase is involved in the closure of phagosomes into intracellular vesicles and not in pseudopod extension, thus placing it downstream of MLCK as well. Protein kinase C may phosphorylate myosin light chain directly, but it does so on sites not included within the peptide we used as MLCK substrate. More recently, the small GTPase Rho has been shown to mediate myosin light chain phosphorylation. However, when PMNLs were treated with Clostridium difficile toxin B to inhibit Rho, phosphorylation of myosin light chain still occurred during phagocytosis (data not shown).

Formation and movement of the pseudopod by actin–myosin interaction occur during various forms of cell movement, including phagocytosis and chemotaxis. MLCK is involved in granulocyte macrophage colony-stimulating factor-stimulated random migration and fMLP-stimulated chemotaxis of PMNLs. Activation of platelets by thrombin, leading to aggregation and granule release, results in diphosphorylation of myosin light chain by MLCK. Phosphorylation of myosin II light chain does not increase during phagocytosis of antibody-opsonized yeast particles by macrophage-like J774 cells; however, HeLa cell phagocytosis appears to involve myosin II. In addition, myosin is phosphorylated during T-lymphocyte receptor capping, implicating MLCK. Thus MLCK activation may be dependent on cell type and function.

During PMNL phagocytosis, as part of the ERK2 pathway MLCK is activated to phosphorylate and activate myosin. Inhibition of phagocytosis by a myosin ATPase inhibitor revealed phagocytosis depends not only on MLCK but on the subsequent myosin–actin interaction arising from the stimulation of myosin ATPase activity. It is highly likely that the myosin ATPase activity translates into propulsive force to form the pseudopod, thereby permitting PMNLs to surround and ingest foreign particles.
References
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