Myosin Light Chain Kinase Regulates Capacitative $\text{Ca}^{2+}$ Entry in Human Monocytes/Macrophages

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Abstract—Monocytes/macrophages are present in all stages of atherosclerosis. Although many of their activities depend to various extents on changes in intracellular $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]), mechanisms regulating [Ca$^{2+}$] in these cells remain unclear. We aimed to explore the role of myosin light chain kinase (MLCK) in $\text{Ca}^{2+}$ signaling in freshly isolated human monocytes/macrophages. Large capacitative $\text{Ca}^{2+}$ entry (CCE) was observed under fura 2 fluoroscopy in human monocytes/macrophages treated with thapsigargin and cyclopiazonic acid. ML-9 and wortmannin, 2 structurally different inhibitors of MLCK, dose-dependently (1 to 100 $\mu$mol/L) prevented CCE and completely did so at 100 $\mu$mol/L, whereas inhibitors of tyrosine kinase and protein kinase C had only partial effects. Western blotting showed that thapsigargin significantly caused myosin light chain phosphorylation, which was almost completely blocked by ML-9 (100 $\mu$mol/L) and wortmannin (100 $\mu$mol/L). ML-9 also dose-dependently (1 to 100 $\mu$mol/L) inhibited this phosphorylation, which was well correlated with its inhibition of CCE. Transfection with MLCK antisense completely prevented CCE in response to thapsigargin and cyclopiazonic acid, whereas MLCK sense had no effect. These data strongly indicate that MLCK regulates CCE in human monocytes/macrophages. The study suggests a possible involvement of MLCK in many $\text{Ca}^{2+}$-dependent activities of monocytes/macrophages. (Arterioscler Thromb Vasc Biol. 2001;21:509-515.)

Key Words: monocytes/macrophages $\bullet$ capacitative $\text{Ca}^{2+}$ entry $\bullet$ myosin light chain kinase

Monocytes/macrophages are present in all stages of atherosclerosis.1–3 Many of their functions depend on changes in intracellular $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]). For example, leukocytes adhering to endothelial cells demonstrate multiple $\text{Ca}^{2+}$ transients;4 the binding of oxidatively damaged red blood cells by macrophages is almost totally Ca$^{2+}$ dependent;5 and oxidized LDL–induced activation of protein kinase C (PKC) leading to macrophage growth also involves a rise in [Ca$^{2+}$].6 In addition, optimal oxidative modification of LDL by monocytes/macrophages requires Ca$^{2+}$ release from intracellular stores and Ca$^{2+}$ entry,7 and LDL receptor–mediated lipoprotein degradation is also Ca$^{2+}$ dependent.8

In many cells, intracellular Ca$^{2+}$ stores are mobilized after either the production of inositol trisphosphate or blockade of the endoplasmic reticulum (ER) Ca$^{2+}$ pump, and it has been postulated that Ca$^{2+}$ store depletion can trigger transmembranous Ca$^{2+}$ entry, the capacitative Ca$^{2+}$ entry (CCE) model.9 Such a mechanism has not been demonstrated in human monocytes/macrophages. Furthermore, although more human homologues of the Drosophila gene products transient receptor potential and transient receptor potential-like are being cloned and have proven to be candidate genes for CCE channels,10 further information is required to determine how intracellular Ca$^{2+}$ store depletion can activate CCE. Numerous second messengers have been implicated in CCE activation, including inositol trisphosphate,11 cGMP,12 a Ca$^{2+}$ influx factor,13 a product of Cytochrome P 450 activity,14 and tyrosine phosphorylation.15,16 We have recently reported that myosin light chain (MLC) kinase (MLCK) inhibitors prevent agonist-stimulated, fluid flow–stimulated, and chloride-sensitive Ca$^{2+}$ entry in endothelial cells.17,18 In monocytes/macrophages, MLCK is implicated in several activities important in atherosclerosis. Thus, MLCK is important for macrophage motility19 and regulates neutrophil migration across the endothelium.20 MLCK activation was recently proven to be critical in cytoskeletal changes resulting in pseudopod formation during phagocytosis.21 Migration and recruitment of macrophages and macrophage-derived foam cells are, in turn, stimulated by increased Ca$^{2+}$ concentration.22 Although these observations, taken together, indicate a likelihood of an involvement of MLCK in Ca$^{2+}$ signaling and thus in many Ca$^{2+}$-dependent activities of these cells, the role of MLCK in monocyte/macroage Ca$^{2+}$ signaling has never been investigated.
In the present study, we have investigated the effects of various inhibitors of MLCK and other protein kinases on CCE and MLC phosphorylation caused by thapsigargin (TG) and cyclopiazonic acid (CPA) in human monocytes/macrophages, and we have tested the effects of MLCK sense and antisense oligonucleotides on the CCE. Our results indicate that MLCK is an important regulator of CCE in human monocytes/macrophages and suggest the possible involvement of the kinase in many Ca\(^{2+}\)-dependent activities of these cells.

Methods

Cell Isolation

The investigation was approved by the institutional ethics committee. Informed consent was obtained from healthy volunteers, and heparinized blood was collected, diluted in PBS containing 2% FCS, and subjected to gravity centrifugation (400g) in Ficoll-Paque isolating solution (Pharma Biotech) for 30 minutes at 18°C. The upper plasma layer was removed, leaving the leukocyte layer undisturbed; this layer was then gently collected and washed several times with PBS containing 2% FCS at 4°C. The final pellets were suspended in RPMI 1640 medium containing 2% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μmol/L streptomycin at a density of 2×10\(^6\) cells/mL, aliquoted onto polybiphenyl dishes fixed on 10×10-mm glass coverslips, and incubated at 37°C under 5% CO\(_2\) for several hours. Before the experiments, samples were washed several times with the same culture medium to remove nonadhering leukocytes, leaving only adherent monocytes/macrophages on glass coverslips. Differential cell counts under Wright’s Giemsa staining showed that >90% of the isolated cells were mononuclear cells. Cell viability was >95%, as determined by trypan blue dye exclusion test.

Western Blotting

MLC phosphorylation in monocytes/macrophages was measured by separation of nonphosphorylated and phosphorylated forms by glyceral-PAGE according to their respective charges of phosphate molecules, followed by electrophoretic transfer of the proteins to a nitrocellulose membrane. The relative amounts of each form were quantified by immunoblotting with an anti-MLC antibody, as described in a previous study with [18]. Immunoblotting by a modification of the method by Persechini et al. was largely increased (Figure 1C). These results clearly implicated CCE in human monocytes/macrophages.

Statistical Analysis

Data are expressed as mean±SD from at least 3 separate experiments. Statistical analysis was performed with the Student t test for unpaired data. Correlation analysis was performed by using GraphPad Prism (GraphPad Software, Inc). A value of P<0.05 was considered significant.

Results

CCE Exists in Human Monocytes/Macrophages

To investigate Ca\(^{2+}\) signaling in human monocytes/macrophages, we used TG, an irreversible inhibitor of the ER Ca\(^{2+}\)-ATPase, and CPA, a reversible inhibitor of the enzyme. Under Ca\(^{2+}\)-free conditions, TG (1 μmol/L) and CPA (100 μmol/L) increased the fluorescence ratios at 340 and 380 nm (F340/380 ratios) very slightly, from 0.88±0.10 and 0.92±0.02 to 1.07±0.06 and 1.10±0.11 after 90 seconds of TG and CPA treatment, respectively (open circles, Figures 1A and 1B). In Ca\(^{2+}\)-containing medium, TG (1 μmol/L) significantly increased the F340/380 ratio from 0.87±0.17 to 4.28±0.39 and 4.00±0.30 after 2 and 8 minutes, respectively (closed circles, Figure 1A). Likewise, CPA (100 μmol/L) largely increased the F340/380 ratio from 0.92±0.12 to 4.10±0.41 and 3.15±0.32 after 3.5 and 8 minutes, respectively (closed circles, Figure 1B). To determine whether CCE was at work, the reversible ER Ca\(^{2+}\)-ATPase inhibitor CPA (100 μmol/L) was first applied in Ca\(^{2+}\)-free medium, which slightly increased the F340/380 ratio. CPA was then washed out, and the cells were kept in Ca\(^{2+}\)-free medium for 5 minutes. When 1 μmol/L Ca\(^{2+}\) was introduced without CPA, the F340/380 ratio was largely increased (Figure 1C). These results clearly implicated CCE in human monocytes/macrophages.

MLCK Inhibitors Prevented Human Monocyte/Macrophage CCE

To investigate the role of MLCK in Ca\(^{2+}\) signaling in human monocytes/macrophages, we first examined the effects of ML-9, a strong MLCK inhibitor, on TG-induced Ca\(^{2+}\) response. Five-minute ML-9 pretreatment did not increase the basal F340/380 ratio but dose-dependently (1 to 100 μmol/L) prevented the TG-induced Ca\(^{2+}\) response, with almost complete inhibition at 100 μmol/L (Figure 2A). In the same manner as used with ML-9, a 30-minute pretreatment with
wortmannin, a different MLCK inhibitor, did not increase the basal F340/380 ratio but dose-dependently (1 to 100 \( \mu \)mol/L) inhibited the TG-induced \( Ca^{2+} \) response, with complete inhibition at 100 \( \mu \)mol/L (Figure 2B).

To confirm that MLCK inhibition could block CCE, CPA was applied for 5 minutes under \( Ca^{2+} \)-free conditions, which slightly raised the F340/380 ratio. CPA was then removed, and ML-9 (100 \( \mu \)mol/L) was applied 5 minutes before 1 mmol/L \( Ca^{2+} \) was added. Even after the addition of \( Ca^{2+} \), the F340/380 ratio did not increase in the presence of ML-9. When ML-9 was removed 7.5 minutes later in \( Ca^{2+} \)-containing medium, the F340/380 ratio increased (Figure 2C).

To test whether MLCK inhibition could affect intracellular \( Ca^{2+} \) store depletion, monocytes/macrophages pretreated with 100 \( \mu \)mol/L ML-9 (Figure 3A and 3C) or 100 \( \mu \)mol/L wortmannin (Figure 3B and 3D) were stimulated with TG (1 \( \mu \)mol/L) or CPA (100 \( \mu \)mol/L) in \( Ca^{2+} \)-free medium. TG and CPA still transiently increased the F340/380 ratios as they did in controls. All in all, these data demonstrate that different MLCK inhibitors prevent CCE without affecting ER \( Ca^{2+} \) release in response to TG and CPA in human monocytes/macrophages.

**MLCK Inhibitors Blocked TG-Stimulated MLC Phosphorylation**

Because a key function of MLCK is to phosphorylate MLC, we investigated the effects of the MLCK inhibitors on TG-induced MLC phosphorylation in monocytes/macrophages (Figure 4). In control conditions, MLC was not phosphorylated (the total extracted MLC being in the non-phosphorylated form); TG (1 \( \mu \)mol/L) increased phosphorylated MLC to as much as 77% of the total extracted MLC. Wortmannin (100 \( \mu \)mol/L) pretreatment for 30 minutes or ML-9 (100 \( \mu \)mol/L) pretreatment for 5 minutes completely inhibited the formation of phosphorylated MLC bodies, which were now only 6% and 0%, respectively, of the total extracted MLC. Wortmannin (100 \( \mu \)mol/L) pretreatment for 30 minutes or ML-9 (100 \( \mu \)mol/L) pretreatment for 5 minutes completely inhibited the formation of phosphorylated MLC bodies, which were now only 6% and 0%, respectively, of the total extracted MLC (Figure 4A). ML-9 dose-dependently (1 to 100 \( \mu \)mol/L) inhibited this MLC phosphorylation (Figure 4B), and the effects of ML-9 to inhibit CCE and MLC phosphorylation were closely correlated (\( P<0.05, r=0.95197 \); Figure 4C). These findings substantiate MLCK involvement in the effects shown by both inhibitors on CCE.
Effects of Other Kinase Inhibitors on CCE

Various protein kinases have been implicated in CCE. Therefore, we compared the effects of inhibitors of MLCK, protein tyrosine kinase (PTK), and PKC on TG- and CPA-induced Ca^{2+} responses. Figure 5 compares the effects on the peak and sustained phases of the Ca^{2+} responses to 1 mM TG (Figure 5A) and 100 mM CPA (Figure 5B) by the MLCK inhibitors ML-9 (100 mM) and wortmannin (100 mM), the PTK inhibitors genistein (100 mM) and herbimycin A (100 mM), and the PKC inhibitors bisindolylmaleimide I (10 mM) and staurosporine (0.3 mM). Pretreatment for 5 minutes with either the PTK or PKC inhibitors significantly, but only partially, inhibited TG- and CPA-induced Ca^{2+} influxes, whereas the MLCK inhibitors almost abolished these influxes. Five minutes was sufficient for the inhibitors to exert their maximal effects, except for wortmannin, which required 30 minutes.

Effects of MLCK Sense and Antisense on TG- and CPA-Induced Ca^{2+} Responses

To provide further independent evidence that MLCK regulates CCE, we transfected human monocytes/macrophages with MLCK sense or antisense oligonucleotides and tested Ca^{2+} responses to TG and CPA in these cells. To confirm that the oligonucleotides were transfected into cells, the 5′ ends were conjugated with FITC, the complexes were transfected, and the cells were observed under a fluorescence microscope. All the cells displayed bright FITC fluorescence, indicating that they all contained MLCK sense and antisense oligonucleotides (please see http://atvb.ahajournals.org). Figure 6 shows the Ca^{2+} responses of control, sense, and antisense cells to 1 mM TG (Figure 6A) and 100 mM CPA (Figure 6C). In antisense cells, Ca^{2+} responses to TG and CPA were completely prevented; there were only small transient rises similar to those observed under Ca^{2+}-free conditions. However, MLCK sense oligonucleotides had no effects on TG- and CPA-induced Ca^{2+} responses. In Ca^{2+}-free medium, small and transient rises in [Ca^{2+}] were still observed after treating MLCK antisense cells with 1 mM TG (Figures 6B) or 100 mM CPA (Figure 6D). A similar transfection protocol with only Superfect Reagent showed no effect on TG- and CPA-induced CCE (data not shown).

Discussion

The ER Ca^{2+}-ATPase inhibitors TG and CPA largely increased [Ca^{2+}], in human monocytes/macrophages in Ca^{2+}-containing medium but did so only very slightly under Ca^{2+}-free conditions. This indicates that the bulk of [Ca^{2+}] rise was due to Ca^{2+} entry. In Figure 1C, Ca^{2+} entry was observed on the restoration of extracellular Ca^{2+} after a short incubation in Ca^{2+}-free conditions after CPA removal. Because the store depletion stimulated by CPA was apparently the only mechanism to trigger the Ca^{2+} entry under this condition, this observation clearly indicates CCE in human monocytes/macrophages. This is similar to Ca^{2+} entry mechanisms in many other cell types.28
Figure 5. Comparative effects of various kinase inhibitors on human monocyte/macrophage CCE. Cells (n=14) were incubated with 2 μmol/L fura 2-AM for 45 minutes at room temperature. Medium contained 1 mmol/L Ca\(^{2+}\). The cells were pretreated for 5 minutes with various kinase inhibitors; wortmannin pretreatment lasted for 30 minutes. Solid and shaded bars indicate peak and plateau Ca\(^{2+}\) responses, respectively, after treatment with TG (A) and CPA (B). Concentrations are as follows: TG, 1 μmol/L; CPA, 100 μmol/L; ML-9, 100 μmol/L; wortmannin (WT), 100 μmol/L; genestein (GEN), 100 μmol/L; herbimycin A (HA), 100 μmol/L; bisindolylmaleimide I (BIM), 10 μmol/L; and staurosporine (STR), 0.3 μmol/L. *P<0.05 vs respective values by TG and CPA; 1P<0.05 vs respective values by ML-9.

The first clue to the involvement of MLCK in the regulation of CCE in human monocytes/macrophages was that ML-9 and wortmannin, structurally different MLCK inhibitors, completely prevented this entry at 100 μmol/L. ML-9 is a potent MLCK inhibitor (K\(_i\) = 3.8 μmol/L) that competes with ATP for binding to the kinase.\(^{29}\) Because ML-9 at high doses can also inhibit PKC (K\(_i\) = 54 μmol/L) and PKA (K\(_i\) = 32 μmol/L), its dose-dependent inhibition of the Ca\(^{2+}\) response was not enough to implicate MLCK in CCE. However, similar effects were observed with structurally unrelated wortmannin, an inhibitor of phosphatidylinositol-3 kinase and MLCK.\(^{50}\) It was used in the present study as an MLCK inhibitor, because TG and CPA mobilize ER Ca\(^{2+}\) not through the activation of phosphatidylinositol-3 kinase. This similarity suggests specificity to MLCK of the effects by both agents to inhibit CCE. The peaks of the Ca\(^{2+}\) responses in Figure 2B seemed delayed, but this cannot be interpreted as an inhibitory effect of wortmannin on store depletion. Ca\(^{2+}\) rises due to store release constitute a very small portion of the rising phases of the Ca\(^{2+}\) response curves. Furthermore, as shown in Figure 3, wortmannin did not affect store mobilization. This delay was not seen with ML-9. In Figure 2C, Ca\(^{2+}\) entry was observed only after the removal of ML-9. This observation and a comparison with Figure 1C clearly show that ML-9 was able to inhibit CCE in monocytes/macrophages.

Further clues to the involvement of MLCK in CCE came from the findings that wortmannin and ML-9 were able to completely inhibit TG-induced MLC phosphorylation at concentrations that completely inhibited TG-induced CCE. IC\(_{50}\) values for ML-9 to block MLC phosphorylation and CCE were also similar, according to Figure 4C. The correlation between the effects of ML-9 to inhibit CCE and MLC phosphorylation further indicates that MLCK was involved in the effect of the compound to inhibit CCE. MLCK phosphorylation by MLCK is linked to cellular contractile activity; therefore, it could be that MLCK activation after Ca\(^{2+}\) store release might rearrange the cytoskeleton, which would open the gate for Ca\(^{2+}\) entry. Nevertheless, MLCK-regulated CCE and MLC phosphorylation could just as likely be independent events; thus, the extent of MLCK phosphorylation would be considered in this regard only as a measure of MLCK activity. In fact, in another series of experiments, calyculin A, a phosphatase inhibitor that should increase MLC-phosphorylated forms by preventing them from being degraded, neither caused any Ca\(^{2+}\) entry nor enhanced the TG-induced Ca\(^{2+}\) responses (authors’ unpublished data, 2000).

Various kinases have been implicated in CCE. Particularly, the PKC inhibitor genistein was found to inhibit CCE in various cells, including fibroblasts,\(^{15}\) lymphocytes,\(^{31}\) platelets,\(^{32}\) and endothelial cells.\(^{32,33}\) PKC involvement in CCE has been controversial. In Xenopus oocytes, activation of PKC was found to inhibit CCE,\(^{34}\) whereas in endothelial cells, we showed that inhibitors of PKC and PKA did not affect agonist-induced Ca\(^{2+}\) entry.\(^{35}\) In the present study, ML-9 and wortmannin abolished TG- and CPA-induced CCE, whereas inhibitors of PKT and PKC had only partial effects. The observations that bisindolylmaleimide I, a strong PKC inhibitor (K\(_i\) = 0.01 μmol/L), PKA (K\(_i\) = 2.0 μmol/L),\(^{36}\) and staurosporine only partially inhibited TG- and CPA-induced CCE make it more likely that the effects seen with ML-9 shown in
Figure 2A were due to MLCK inhibition. These data suggest that MLCK may play a more important role than do PTK and PKC in CCE in human monocytes/macrophages. MLCK has several potential phosphorylatable sites that can be phosphorylated by other protein kinases, including PKC and PTK. In fact, the PTK inhibitor genistein was shown to reduce MLCK activity in MLCK immunoprecipitates, and it has been suggested that genistein-sensitive tyrosine kinase activities are involved in thrombin-mediated MLCK activation and MLCK phosphorylation. It is possible, then, that MLCK, partially influenced by PKC and PTK, lies downstream from these 2 kinases in the CCE signaling cascade and plays a more decisive role in the regulation of this cascade in human monocytes/macrophages. This hypothesis seems to explain the proportional contribution of PTK, PKC, and MLCK to the coupling of internal store depletion and transmembranous Ca\(_2\)\(^+\) entry in these cells.

The experiments above suggest that MLCK may be important in CCE activation in human monocytes/macrophages and that MLCK may be more important than PTK and PKC in this regard. Nevertheless, because all the inhibitors used significantly inhibited CCE, they could be accomplishing this by some nonspecific effects on the cells. The observations that transfection with MLCK antisense completely prevented CCE but that MLCK sense had no effect provide solid independent evidence that MLCK plays a crucial role in the regulation of CCE in human monocytes/macrophages. The coupling role is clear because neither the MLCK inhibitors nor MLCK antisense affected the emptying of the Ca\(_2\)\(^+\) store by TG and CPA.

In conclusion, we have provided solid evidence that MLCK has a crucial role in the regulation of CCE in human monocytes/macrophages. Together with our previous findings in endothelial cells, the present study suggests a possible involvement of MLCK in the pathogenesis of atherosclerosis, the initiation of which involves both of these cell types. The present study suggests the involvement of MLCK in many Ca\(_2\)\(^+\)-dependent activities of these cells. Further investigations are under way to test these hypotheses.

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