Ca\(^{2+}\) Influx through L-type Ca\(^{2+}\) Channels Controls the Trailing Tail Contraction in Growth Factor-induced Fibroblast Cell Migration*

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Growth factor-induced cell migration underlies various physiological and pathological processes. The mechanisms by which growth factors regulate cell migration are not completely understood. Although intracellular elevation of Ca\(^{2+}\) is known to be critical in cell migration, the source of this Ca\(^{2+}\) elevation and the mechanism by which Ca\(^{2+}\) modulates this process in fibroblast cells are not well defined. Here we show that increase of cellular Ca\(^{2+}\) through Ca\(^{2+}\) influx, rather than Ca\(^{2+}\) release from intracellular stores, is essential for growth factor-induced fibroblast cell migration. Voltage-gated L-type Ca\(^{2+}\) channels, previously known to exist in excitable cells such as neurons and muscle cells, are shown here to be present in fibroblasts as well. Furthermore, these channels are responsible for the Ca\(^{2+}\) influx. L-type Ca\(^{2+}\) channel inhibitors block growth factor-induced Ca\(^{2+}\) influx and fibroblast cell migration. One mechanism by which Ca\(^{2+}\) signals control cell migration is to regulate the contraction of the trailing edge of migrating fibroblasts; this process is controlled by the small GTPase Rho in fast migrating cells such as leukocytes. Downstream of Ca\(^{2+}\), both calmodulin and myosin light chain kinase, but not calcineurin, are involved leading to phosphorylation of the myosin light chain at the trailing end. Thus, trailing edge contraction is critically regulated by Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels in growth factor-induced fibroblast cell migration.

Growth factor-induced cell migration plays essential roles in organism development, physiological functions, and pathological disease processes (1, 2). In wound healing, fibroblasts migrate into the wound site. Once within the wound site, fibroblasts proliferate and lay down a new collagen-rich connective tissue matrix (3). Vascular smooth muscle cell migration induced by platelet-derived growth factor (PDGF) and other factors contributes to the pathophysiology of intimal hyperplasia and is an essential component of the intimal process that leads to recurrent stenosis (4). The invasion and metastasis of tumor cells also require cell migration (5). Solid tumors, which account for more than 85% of cancer mortality, require blood vessels for growth, and many new cancer therapies are directed against the tumor vasculature, a process involving growth factor-induced cell migration (6).

Ca\(^{2+}\) is a critical second messenger for a wide range of physiological processes, including the electrical excitability of neurons, muscle contraction, cellular secretion, and gene expression (7). Ca\(^{2+}\) also plays a regulatory role in cell migration such as in neutrophils, eosinophils, smooth muscle cells, and neurons (8–12). Cell migration is a sequential and interrelated multistep process (2). It involves the formation of lamellipodia/membrane protrusion at the front edge, cycles of adhesion and detachment, cell body contraction, and tail retraction. In migrating cells, Ca\(^{2+}\) concentration is high at the rear end and low at the front leading edge (9, 12–14).

Increase of cellular Ca\(^{2+}\) levels is brought about by either Ca\(^{2+}\) release from intracellular stores or Ca\(^{2+}\) influx through plasma membrane Ca\(^{2+}\) or other cation channels (7). Both Ca\(^{2+}\) release and Ca\(^{2+}\) influx have been linked to cell migration depending on cell-types and stimuli (10, 12, 15, 16). In some cases, it is the induction of a Ca\(^{2+}\) transient that is important for cell migration irrespective of the Ca\(^{2+}\) source (8, 16). Among the membrane channels are the voltage-gated L-type Ca\(^{2+}\) channels, which selectively allow the flow of Ca\(^{2+}\) ions down an electrochemical gradient, from a high concentration outside the cell to a low concentration inside the cell, to increase cytosolic Ca\(^{2+}\) level (17). These L-type Ca\(^{2+}\) channel proteins are expressed in excitable cells such as neurons and cardiac myocytes (17). Indeed, L-type Ca\(^{2+}\) channels have been genetically implicated in controlling neuronal migration in Caenorhabditis elegans (18) and axonal guidance in Xenopus spinal neurons (19). Although cellular Ca\(^{2+}\) is critical for fibroblast cell migration, the source of the Ca\(^{2+}\) increase and the mechanism by which Ca\(^{2+}\) plays its role in controlling fibroblast cell migration are not clear.

Here we use mouse embryonic fibroblasts (MEFs) to study the potential role of voltage-gated L-type Ca\(^{2+}\) channels in growth factor-induced cell migration. We found that Ca\(^{2+}\) release from intracellular stores is not essential for PDGF-induced MEF cell migration. Rather, PDGF-induced cell migration requires Ca\(^{2+}\) influx. This Ca\(^{2+}\) influx is mediated by L-type Ca\(^{2+}\) channels present in MEF cells. Furthermore, downstream of Ca\(^{2+}\), calmodulin and myosin light chain kinase (MLCK) mediate the phosphorylation of myosin light chain (MLC) at the trailing end of migrating fibroblasts, leading to the retraction of the rear edge during cell migration.

EXPERIMENTAL PROCEDURES

Cells and Reagents—MEF cells and human MDA-MB-231 breast tumor cells were cultured in DMEM supplemented with 10% FBS and 2 mM glutamine. Mouse 4T1 breast tumor cells were cultured in RPMI 1840 medium supplemented with 10% FBS. Human umbilical vein endothelial cells were cultured in Medium 199 supplemented with 10% FBS. Phospholipase C (PLC)γ1−/− and PLCγ1−/−+PLCγ1 MEF cells were generously provided by Dr. G. Carpenter (Vanderbilt University).
and were cultured in DMEM supplemented with 10% FBS and 2 mM glutamine (20). PDGF-BB was from Oncogene Research Products. BAPTA-AM (1,2-bis [aminophenoxo] ethane-N,N',N'-tetraacetic acid) buffer solutions were from Calbiochem.

In Vitro Wound-healing Cell Migration Assay—Cell migration assays were performed as described previously (21). Cells were allowed to form a confluent monolayer in a 24-well plate coated with gelatin before wounding. The wound was made by scraping a conventional pipette tip across the monolayer. The migration was induced by adding medium supplemented with or without 10% FBS, 20 ng/ml PDGF or 20 ng/ml epidermal growth factor. For MEF cells, it typically took 8–10 h for the wound to close. When the wound for the positive control closed, cells were fixed with 3.7% formaldehyde and stained with crystal violet staining solution.

Boyden Chamber Cell Migration Assay—MEF cells (5 × 10^4) suspended in DMEM were added to the upper chamber of an insert coated with gelatin (6.5-mm diameter, 8-μm pore size, Becton Dickinson), and the chamber was placed in 24-well dishes containing DMEM with or without 10% FBS, 40 ng/ml PDGF or 40 ng/ml epidermal growth factor. When used, inhibitors were added to both chambers. Migration assays were carried out for 4 h and cells were fixed with 3.7% formaldehyde. Cells were stained with crystal violet staining solution, and cells on the upper side of the insert were removed with a cotton swab. Three randomly selected fields (10× objective) were photographed, and the migrated area was measured. The migration was expressed as either the average number of migrated cells in a field or as percentage of migrated cells.

Percentage was calculated using the formula P = 100 × (M – M₀)/M₀, where P is the percentage of migrated cells, M is the number of migrated cells, M₀ is the number of migrated cells in negative controls (DMEM only), and M is the number of migrated cells in positive controls (with 10% FBS or PDGF).

Ca²⁺ Assay and Western Blots—The Ca²⁺ assay was conducted as previously described (22). Cells were plated onto gelatin-coated 96-well plates (~50,000 cells/well) 1 day before the experiments. The standard solution for calcium assays consisted of 20 mM HEPES (pH 7.4), 130 mM NaCl, 2 mM CaCl₂, 5 mM KCl, 10 mM glucose, 0.45 mM KH₂PO₄, 0.4 mM NaH₂PO₄, 1.2 mM MgSO₄, 1.2 mM MgCl₂, 4.2 mM NaHCO₃, 2.5 mM probenecid (Sigma), and 0.1% bovine serum albumin (Sigma). The calcium-sensitive dye, Fluo-3 (Molecular Probes), was dissolved in dimethyl sulfoxide (Me₂SO), mixed with an equal volume of 20% Pluronic 127 (Sigma), and diluted in standard solution plus 1% bovine serum albumin to a final concentration of 4 μM. Cells were washed with standard solution and incubated with 100 μM of 4 μM Fluo-3/Me₂SO for 45 min at 22 °C followed by 15 min at 37 °C. Cells were then washed three times with standard solution (200 μl/well) on ice. Finally, 100 μl of standard solution was added per well, and cells were incubated at 37 °C for 15 min before the assay. The change in fluorescence was monitored using a Fluorescent Ascent FL fluorometer. Western blots were performed as described previously (23).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described previously (24). Cells cultured on gelatin-coated coverslips were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 3 min, and blocked with 1% bovine serum albumin in PBS for 1 h. Cells were then incubated with primary antibodies at appropriate dilutions (1:100 for rabbit anti-pSer-19 MLC (from Cell Signaling); 1:4000 for rabbit anti-L-type Ca²⁺ channel blockers, Ni²⁺ and La³⁺, that block Ca²⁺ channels without affecting extracellular free Ca²⁺ concentrations. As shown in Fig. 2, E and F, both Ni²⁺ and La³⁺ blocked PDGF and serum-induced cell migration. Therefore, these data demonstrated that Ca²⁺ influx is critical for PDGF-induced cell migration. Furthermore, the abolishment of serum-induced MEF cell migration by EGTA, Ni²⁺, and La³⁺ indicated that Ca²⁺ influx might play a general regulatory role in cell migration. To test this hypothesis, the effect of Ni²⁺ and EGTA on cell migration was examined with mouse 4T1 breast tumor cells, human MDA-MB-231 breast tumor cells, and human umbilical vein endothelial cells. As shown in Fig. 2, G-I, Ni²⁺ and EGTA blocked serum-induced migration of all these three types of cells (data with EGTA not shown). These results suggest that Ca²⁺ influx is generally required for normal cell as well as cancer cell motility.

RESULTS

Ca²⁺ Release Is Not Essential for PDGF-induced MEF Cell Migration—In MEF cells, stimulation with PDGF induced cell migration, as measured by both in vitro wound-healing assay (Fig. 1A) and Boyden chamber assay (Fig. 1B). This migration was blocked by treatment with the membrane-permeable Ca²⁺ chelator, BAPTA-AM, indicating that Ca²⁺ plays an indispensable role in PDGF-induced MEF cell migration (Fig. 1B). However, Ca²⁺ elevation by itself was not sufficient because the Ca²⁺ ionophore, ionomycin, treatment did not induce cell migration (data not shown). To investigate the source of Ca²⁺ increase in fibroblasts after PDGF treatment, we examined the significance of Ca²⁺ release and Ca²⁺ influx in fibroblast cell migration. Growth factor receptor tyrosine kinase-induced Ca²⁺ responses usually consist of two phases, an early transient peak that is due to Ca²⁺ release from internal stores, and a late sustained plateau that is due to Ca²⁺ influx (7). Ca²⁺ release is initiated by the binding of inositol 1,4,5-triphosphate to its receptor on the endoplasmic reticulum. Inositol 1,4,5-triphosphate is generated by the hydrolysis of phosphatidyl inositol 4,5-bisphosphate by PLC. In response to growth factors, PLCγ is responsible for the Ca²⁺ release (25). In fibroblast cells, PLCγ1 is the main expressed PLCγ isoform (20). Indeed, in PLCγ1⁻/⁻ fibroblasts, PDGF-induced Ca²⁺ release from internal stores was abolished, whereas Ca²⁺ influx was intact (Fig. 1, C and D). This Ca²⁺ response deficiency was because of the absence of PLCγ1 because re-expression of PLCγ1 in PLCγ1⁻/⁻ cells restored Ca²⁺ release after PDGF stimulation (Fig. 1E). If Ca²⁺ release is essential for PDGF-induced fibroblast cell migration, PLCγ1⁻/⁻ cells should be defective in migration. However, we observed that PLCγ1⁻/⁻ cells still migrate under the stimulation of PDGF (Fig. 1, F and G). Consistent with a previous report with epidermal growth factor (26), the PDGF-induced migration of PLCγ1⁻/⁻ cells and PLCγ1-rescued cells was comparable (Fig. 1, H and I). These data demonstrate that Ca²⁺ release is not essential for PDGF-induced fibroblast cell migration.

Ca²⁺ Influx Is Required for Cell Migration—Next we investigated whether Ca²⁺ influx plays a role in PDGF-induced cell migration. Chelation of extracellular Ca²⁺ with EGTA completely blocked both PDGF and serum induced MEF cell migration (Fig. 2, A–D). This EGTA abolishment of cell migration was reversed by the addition of equal molar Ca²⁺ into the medium (data not shown). To further confirm the requirement for Ca²⁺ influx, we used the general Ca²⁺ channel blockers, Ni²⁺ and La³⁺, that block Ca²⁺ channels without affecting extracellular free Ca²⁺ concentrations. As shown in Fig. 2, E and F, both Ni²⁺ and La³⁺ blocked PDGF and serum-induced cell migration. Therefore, these data demonstrated that Ca²⁺ influx is critical for PDGF-induced cell migration. Furthermore, the abolishment of serum-induced MEF migration by EGTA, Ni²⁺, and La³⁺ indicated that Ca²⁺ influx might play a general regulatory role in cell migration. To test this hypothesis, the effect of Ni²⁺ and EGTA on cell migration was examined with mouse 4T1 breast tumor cells, human MDA-MB-231 breast tumor cells, and human umbilical vein endothelial cells. As shown in Fig. 2, G-I, Ni²⁺ and EGTA blocked serum-induced migration of all these three types of cells (data with EGTA not shown). These results suggest that Ca²⁺ influx is generally required for normal cell as well as cancer cell motility.

L-type Ca²⁺ Channels Mediate Ca²⁺ Influx in PDGF-induced MEF Cell Migration—In studying which type of Ca²⁺ channels were responsible for this Ca²⁺ influx, we found that the voltage-gated L-type Ca²⁺ channels were essential for PDGF-induced Ca²⁺ influx and cell migration of MEF cells. First, although L-type Ca²⁺ channel proteins are mainly expressed in excitable cells such as neurons and muscle cells, these channel proteins (the α₁C subunits) are also present in MEF cells as shown by Western blot (Fig. 3A). Immunostaining of MEF cells with anti-L-type Ca²⁺ channel antibodies revealed membrane staining (Fig. 3B). L-type Ca²⁺ channel proteins are uniformly distributed on the plasma membrane (including the front as well as the tail of a migrating MEF cell). The staining at the tail membrane was overshadowed by the strong perinuclear
Depolarization of MEF cell membrane potential with 150 mM KCl expressed in MEF cells, but they are functional also. Specific L-type Ca\(^{2+}\) channels are generally involved in growth factor-induced fibroblast cell migration (27), we first examined whether Ca\(^{2+}\) acts upstream or downstream of, or in parallel to, Rac. MEF cells were infected with retroviruses carrying Rac1(G12V), a constitutively active mutant of Rac1. Compared with cells infected with the control retroviral vector, expression of Rac1(G12V) significantly promoted MEF cell migration (Fig. 4A). This Rac1(G12V)-promoted cell migration was abolished by Ca\(^{2+}\) influx blocker Ni\(^{2+}\) (Fig. 4B). Furthermore, 1 h after making the wound in an in vitro wound-healing assay, lamellipodia (a Rac-dependent phenomenon) were seen along the wound edge of EGTA-, Ni\(^{2+}\)-, or nimodipine-treated cells in the presence of PDGF (Fig. 4C; data with Ni\(^{2+}\) not shown). These data suggested that the Ca\(^{2+}\) signal acts downstream of or in parallel to Rac and that Ca\(^{2+}\) is not required for the formation of lamellipodia and membrane protrusions at the leading edge of migrating fibroblasts.

Because calmodulin is a major mediator of Ca\(^{2+}\) signaling, we tested whether calmodulin mediates Ca\(^{2+}\) regulation of cell migration. Treatment of MEF cells with calmodulin antagonist W7 significantly inhibited PDGF- and serum-induced cell migration (Fig. 4, D and E). The role of calmodulin in cell migration was further confirmed by stable expression of a calmodulin binding peptide in MEF cells. Expression of a calmodulin bind-
ing peptide blocked PDGF- and serum-induced cell migration (Fig. 4, F and G). Among the calmodulin effectors, calcineurin was reported to be involved in integrin turnover. Inhibition of calcineurin blocked neutrophil migration, and this blockage could be reversed by the addition of integrin antibodies (28, 29). However, in MEF cells, calcineurin inhibitor cyclosporin A had no effect on PDGF-induced MEF migration, whereas control experiments showed that at this concentration cyclosporin A inhibited calcineurin activity effectively (data not shown).

Another calmodulin target is MLCK, which phosphorylates the regulatory light chain of myosin II MLC and thus activates myosin II (30). The essential role of myosin II in actin cytoskeletal rearrangement and cell migration has long been appreciated (31). The contraction of actomyosin filaments contributes to the cell body movement and tail retraction in migrating cells (1, 32). Myosin II deficiency and myosin II inhibitors impaired Dictyostelium and neutrophil migration (33, 34). Moreover, in a number of cell types including leukocytes, lymphocytes, and Dictyostelium, myosin II has been reported to localize to the rear end of migrating cells (33, 34). Rho and its effector Rho

**Fig. 2. Ca²⁺ influx is required for cell migration.** A and B, in vitro wound-healing assay (A) and Boyden chamber assay (B) showed that PDGF-induced MEF cell migration was blocked by addition of EGTA to the culture medium. C and D, the in vitro wound-healing assay (C) and Boyden chamber assay (D) showed that serum (10% FBS)-induced MEF cell migration was blocked by the addition of EGTA to the culture medium. E, the in vitro wound-healing assay showed that both PDGF- and serum-induced MEF cell migration was blocked by addition of Ni²⁺ to the culture medium. F, the in vitro wound-healing assay showed that both PDGF- and serum-induced MEF cell migration was blocked by addition of La³⁺. G–I, Ni²⁺ blocked serum-induced 4T1 mouse breast tumor cell migration (G), human MDA-MB-231 breast tumor cell migration (H), human umbilical vein endothelial cell migration (I). Data are representative of three experiments.
kinase are considered to be the major regulators of myosin II in cell migration. Rho kinase could activate myosin II by either directly phosphorylating MLC, or by inhibiting MLC phosphatase thereby indirectly elevating the MLC phosphorylation level (35). However, the role of Rho and Rho kinase in cell migration is cell-type-dependent (36). In fast migrating cells such as macrophages and neutrophils, Rho and Rho kinase appear to be required for cell polarization and migration (35). In slow migrating cells, such as fibroblasts, Rho and Rho kinase appear to inhibit migration. Indeed, Rho inhibitor C3 toxin did not decrease MEF cell migration (data not shown), and Rho kinase inhibitor Y-27632 even promoted MEF cell migration (Fig. 5A), confirming that Rho and Rho kinase are not essential for promoting fibroblast migration. Therefore, we examined the role of MLCK in PDGF-induced cell migration. Treatment of MEF cells with MLCK inhibitor ML-7 markedly inhibited PDGF- and serum-induced migration (Fig. 5, B and C). These data suggested that MLCK acts downstream of calmodulin mediating Ca^{2+} control of cell migration.

To further investigate the relationship between Ca^{2+} influx and MLC phosphorylation, we examined the distribution of phosphorylated MLC in migrating cells. As shown in Fig. 6A, there were two areas of phosphorylated-MLC staining in polarized migrating cells, postlamellipodia staining and trailing tail staining. Strong staining of phosphorylated-MLC was observed in the actin assembly immediately behind the lamellipodia of polarized migrating cells (Fig. 6A). The other area of phosphorylated-MLC staining was in the rear end of the cell body, especially the trailing tail of polarized cells (Fig. 6A). This staining pattern of phosphorylated-MLC was also confirmed by the ratio imaging of phosphorylated-MLC over total proteins (Fig. 6A). During migration, cells can adopt two morphologies. In the first morphology, membrane protrusion at the leading edge and strong adhesion at the rear force cells to adopt an elongated shape with a trailing tail (Fig. 6A). In these cells, extension of the front edge occurs as a distinct phase from retraction of the rear end (37). When elongation tension reaches a threshold, the trailing tail detaches from the substratum and retracts (37). After retraction, the cell adopts the second, fan-shaped morphology (Fig. 6B). The trailing tail phosphorylated-MLC staining was only observed in the elongated migrating cells. In the fan-shaped cells, only postlamellipodia staining of phosphorylated-MLC remained, but no or weak trailing tail staining was observed in most cells (Fig. 6C). In the untreated control group (Fig. 6A), 87% (41 of 47) of cells showed strong trailing edge staining. Moreover, treatment with MLCK inhibitor ML-7 reduced the staining of phosphorylated-MLC, whereas the remaining 17% (8 of 47) of cells showed no or weak tail staining of phosphorylated-MLC. In Ni^{2+}-treated group (Fig. 6C), 39 of the 47 observed elongated cells showed strong trailing tail staining with anti-phosphorylated MLC antibody, whereas the remaining 6 cells showed weak trailing tail staining of phosphorylated-MLC.

**DISCUSSION**

Our results have demonstrated that L-type Ca^{2+} channels are required for MEF cell migration. There are two unexpected findings here. One is that voltage-gated L-type Ca^{2+} channels play an important role in fibroblast cells. Previously, voltage-gated L-type Ca^{2+} channel proteins were thought to be mainly expressed in excitable cells such as neurons and muscle cells (38). Our data revealed that these channel proteins are expressed in fibroblast cells and play an essential role in fibro-
Ca²⁺ Channel in Fibroblast Cell Migration

Recent studies have shown that L-type Ca²⁺ channels are essential for T lymphocyte functions in mice (40). These recent studies revealed a much wider expression pattern for L-type Ca²⁺ channel proteins, consistent with our observation here.

The second unexpected finding reported here is that L-type Ca²⁺ channels, instead of Rho and Rho kinase, control the contraction of the trailing end of migrating fibroblasts. Rho and Rho kinase have long been suggested to be responsible for the actomyosin contraction at the trailing tail in fast migrating cells such as neutrophils and macrophages (35, 36). Our data clearly demonstrate that Ca²⁺ influx through L-type Ca²⁺ channels is responsible for the trailing tail MLC phosphorylation in slow moving fibroblasts. It is possible that in other slow migrating cell types such as epithelial cells, Ca²⁺ influx rather than Rho and Rho kinase might be responsible for the rear end contraction. Consistent with this possibility, we have shown that mouse breast tumor cells and human breast tumor cells (both are epithelial origin) were sensitive to Ca²⁺ influx blockers (Fig. 2).

Through calmodulin and MLCK, Ca²⁺ influx modulates the phosphorylation of MLC at the trailing tail of migrating cells, and thus the actomyosin contractile force that is required for cell body movement and trailing tail detachment. MLC phosphorylation has also been proposed to control membrane protrusions (41). Furthermore, we have also found that Ca²⁺/calmodulin-dependent protein kinase II, another direct effector of calmodulin, is also required for PDGF-induced MEF cell migration (data not shown). Indeed, in vascular smooth muscle cells, it was claimed that Ca²⁺/calmodulin-dependent protein kinase II might be the major mechanism by which Ca²⁺ modulates cell migration (42). At the present time, it is not clear what the downstream substrate of Ca²⁺/calmodulin kinase II is in the MEF cell migration process. In addition to this increased cytoskeletal contractility, Ca²⁺ has also been implicated to induce the disassembly of cell substratum adhesion and to activate gelsolin and other Ca²⁺-sensitive actin-binding proteins (43, 44).

The connection between PDGF stimulation and the opening of L-type Ca²⁺ channels remains to be determined. Similar to those in T lymphocytes (40), L-type Ca²⁺ channels in fibroblast cells retain their voltage dependence (sensitive to membrane depolarization by KCl). However, the opening of these channels

Ca²⁺ Channel in Fibroblast Cell Migration
in T lymphocytes and fibroblast cells is controlled by receptor signals. In neurons, stimulation of insulin-like growth factor 1 activates L-type Ca\textsuperscript{2+} channels through a pathway involving phosphatidylinositol 3-kinase, Akt, and tyrosine kinase Src leading to channel protein phosphorylation (45). Whether such a pathway operates in MEF cells is not clear. Additionally, in Xenopus spinal neurons, cAMP and cGMP have been shown to modulate the activity of L-type Ca\textsuperscript{2+} channels in axonal guidance through protein kinases A and G (19). Moreover, because L-type Ca\textsuperscript{2+} channels are also directly or indirectly sensitive to mechanical stretching (46), tension could serve as a means to open L-type Ca\textsuperscript{2+} channels. It has been reported that tension generated in migrating cells could open Ca\textsuperscript{2+} channels and induce a transient increase in local Ca\textsuperscript{2+} concentration (16). Noticeably, Ca\textsuperscript{2+} influx-induced trailing tail phosphorylation of MLC was only observed in the migrating cells under high tension, i.e. elongated migrating cells, whereas in the cells with reduced tension, i.e. fan-shaped migrating cells, the trailing tail staining disappeared. Therefore, because of membrane protrusion at the leading edge and strong adhesion at the rear induced by PDGF, the tension increases during migration. Tension, possibly with other signals initiated from PDGF, activates Ca\textsuperscript{2+} channels, including L-type Ca\textsuperscript{2+} channels in MEF cells. When the tension reaches a threshold, the trailing tail detaches from the substrate and retracts. Consequently, the decrease in membrane tension leads to the closure of Ca\textsuperscript{2+} channels and the start of the next round of migration turnover. Alternatively, growth factor signals trigger the opening of Ca\textsuperscript{2+} channels that lead to the generation of tension.

In migrating MEF cells, there are two areas with strong staining of phosphorylated MLC, post-lamellipodia and trailing tail. A similar bimodal staining pattern of phosphorylated MLC was observed in motile REF-2A fibroblast cells (47). We have shown that Ca\textsuperscript{2+} influx-activated MLCK is responsible for the phosphorylation of MLC at the trailing tail, without an effect on the phosphorylation of MLC at the postlamellipodia. Then what is responsible for phosphorylating MLC at the postlamellipodia? Surprisingly, we found that inhibition of Rho kinase blocked the phosphorylation of MLC at the postlamellipodia, without affecting the phosphorylation of MLC at the trailing tail.\textsuperscript{2} As we showed in Fig. 5A, Rho kinase inhibitor Y-27632 actually increased MEF cell migration. These results indicate that the phosphorylation of MLC at the postlamellipodia does not play an essential role for MEF cell migration. It is possible that the phosphorylation of MLC at the postlamellipodia functions in focal adhesion formation (41). During cell migration, these focal adhesions need to be formed and detached repeatedly. Therefore, Rho kinase inhibitors might enhance this focal adhesion cycle (possibly through the activation of myosin phosphatase), thus leading to a slight increase of cell migration (41). Focal adhesion turnover is not solely controlled by MLC phosphorylation because abolishment of phosphorylation of MLC (by Rho kinase inhibitors) at the postlamellipodia did not block cell migration.

The fact that inhibition of Ca\textsuperscript{2+} influx blocked both PDGF- and serum-induced cell migration indicates that Ca\textsuperscript{2+} influx plays a general and essential role for cell migration. Most importantly, we have shown here that blocking Ca\textsuperscript{2+} influx inhibited the migration of tumor cells (Fig. 2, G and H). Therefore, inhibitors of Ca\textsuperscript{2+} influx should be explored as blockers of tumor metastasis in cancer therapies.

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Ca\(^{2+}\) Channel in Fibroblast Cell Migration
