CMLS, Cell. Mol. Life Sci. 62 (2005) 65–76
1420-682X/05/010065-12
DOI 10.1007/s00018-004-4391-6

CMLS Cellular and Molecular Life Sciences

Research Article

Myosin regulation in the migration of tumor cells and leukocytes within a three-dimensional collagen matrix

P. Bastian, K. Lang, B. Niggemann, K. S. Zaenker and F. Entschladen*

Institute of Immunology, Witten/Herdecke University, Stockumer Strasse 10, 58448 Witten (Germany),
Fax +49 2302 669 158, e-mail: frankent@uni-wh.de

Received 31 August 2004; accepted 26 October 2004

Abstract. The migration of cells is a complex regulatory process which results in the generation of motor forces through the reorganization of the cytoskeleton. Here we present a comparative study of the expression and involvement of myosin in the regulation of the physiological migration of leukocytes and the pathological migration of tumor cells. We show that the involvement of myosin in the migration is distinct in these two cell types. In leukocytes, the activity of non-muscle myosin II is essential for both the spontaneous (matrix-induced) migration and the migration induced by ligands to G protein-coupled receptors, i.e. chemokines and neurotransmitters. In contrast, spontaneous tumor cell migration is largely independent of non-muscle myosin II activity, whereas the norepinephrine-induced migration is completely inhibited by either direct inhibition of non-muscle myosin II or of the kinases phosphorylating the myosin light chain, namely ROCK or the calcium/calmodulin-dependent myosin light-chain kinase.

Key words. Cell migration; leukocyte; tumor cell; signal transduction; non-muscle myosin II; myosin light chain.

The interaction of myosin and actin is the essential event for the contraction of muscle cells. For the movement of single eukaryotic cells, e.g. leukocytes in the immune response, fibroblasts in wound healing, and tumor cells in invasion and metastasis development, the regulation of actin has been elucidated in detail, e.g. its involvement in focal adhesion contacts [1] and pseudopod development [2], but considerably less attention has been paid to the regulation of myosin. However, although there is a lack of molecular data, current cell migration models consider myosin to play a crucial role in the generation of migration forces [3–5].

Presently, 18 myosin isotype families are known, and 40 myosin genes belonging to 12 of these families are expressed in humans [6]. Myosin II isotypes are the conventional motor proteins; they are ubiquitously expressed in muscle and non-muscle cells [7]. Myosins not only play a role in cell contraction and cytokinesis, they have other very diverse functions: myosin subtypes I and Va function as motor proteins during particle uptake by macrophages [8], and myosin Va plays a role in melanosome transport in melanocytes [9]. Other myosins are involved in the processes of hearing and seeing [10]. Besides myosin II, other myosin isotypes have been shown to be involved in cell migration: myosin VI is required for the migration of border cells in *Drosophila melanogaster* [11], and for the migration of fibroblasts [12] and ovarian cancer cells [13]. Myosin VIIa plays an important role in dynamic cell adhesion during migration [14].

In striated muscles, the troponin-tropomyosin complex regulates the interaction of myosin with actin and thereby the force-generation [15]. In smooth muscle cells, this complex is not expressed [16]. The activity of myosin II in these cells as well as in non-muscle cells is regulated

* Corresponding author.

by the phosphorylation of the myosin light chain (MLC). Here, two classes of enzymes are the key players: first, the myosin light chain kinase (MLCK), which is activated by calcium-calmodulin (CaM) [17], and second, the Rho-kinase/Rho-dependent coiled-coil kinase (ROCK) [18]. The Rho-kinase/ROCK and related kinases such as the citron kinase are activated by Rho [18, 19], which is a monomeric G protein (a small GTPase). The activation of myosin by Rho is an important signaling event in cytokinesis, the final step in cell division [20].

Smith and co-workers have recently shown the spatially segregated function of the MLCK and ROCK-I in the integrin-mediated migration of T lymphocytes [21]. Similarly, these two types of kinases were found to regulate myosin differentially in a hepatic stellate cell line [22], and inhibitors of MLCK block the invasion of human pancreatic cancer cell lines [23]. Furthermore, MLCK is involved in the turnover of focal adhesions [24]. Focal adhesions are a key structural element in the migration of slow-moving cells such as fibroblasts and tumor cells [3]. Here, we investigated the regulation of myosin activity in the migration of breast and prostate carcinoma cells compared to the regulation in leukocytes, namely T lymphocytes and neutrophil granulocytes. From an understanding of the migratory mechanisms in leukocytes and tumor cells and their differences, selective inhibition of tumor cell migration as the prerequisite for invasion and metastasis development might be possible.

Materials and methods

Cell isolation and cell culture

Human cytotoxic T lymphocytes (CTLs) and neutrophil granulocytes were isolated from peripheral blood [25]. In the first isolation step, the peripheral blood mononuclear cell (PBMC) fraction was separated from the erythrocyte/neutrophil granulocyte fraction by density-gradient centrifugation using Ficoll-Hypaque (ICN, Meckenheim, Germany). CTLs were isolated from the PBMC fraction by a positive selection using immunomagnetic beads coated with mouse anti-human CD8 antibody (Dynabeads; Dynal, Hamburg, Germany). The cell-bound beads were detached with polyclonal anti-mouse Fab Abs (Detachabead, Dynal). Isolated CTLs were maintained overnight in RPMI culture medium (PAA, Linz, Austria), 10% heat-inactivated fetal calf serum (PAA) and 1% penicillin/streptomycin (50 U/ml and 50 µg/ml; Gibco, Eggenstein-Leopoldshafen, Germany) at 37 °C in a humidified atmosphere containing 5% CO_2.

The pellet of the density gradient centrifugation containing the erythrocyte/neutrophil granulocyte fraction was mixed with platelet-depleted serum of the same blood donor, and subsequently diluted 1:1.3 with a high molecular-weight dextran solution (Macrodex; Fresenius, Bad Homburg, Germany) containing 0.01 M EDTA. After 3 h the supernatant containing the neutrophil granulocytes was separated from the pellet containing the erythrocytes. Remaining erythrocytes were removed by a hypotonic lysis with 0.3% sodium chloride for 2 min on ice. The purified neutrophil granulocytes were used immediately after isolation.

The human breast carcinoma cell line MDA-MB-468 (ATCC, Rockville, Mass.) was cultured in Leibovitz's L-15 culture medium (PAA) containing 10% heat-inactivated fetal calf serum (PAA), and the human prostate carcinoma cell line PC-3 (DSMZ, Braunschweig, Germany) was cultured in HAM's and RPMI culture medium (1:1, both obtained from PAA) containing 10% heat-inactivated fetal calf serum. Both cell lines were kept in a 37 °C humidified atmosphere with 5% CO_2.

Cell migration assay and data analysis

The migratory activity of the cells within three-dimensional collagen lattices was recorded by time-lapse videomicroscopy and analyzed by computer-assisted cell tracking [25, 26]. In brief, 2×10^5 leukocytes or 6×10^4 tumor cells were mixed with 150 µl carbonate-buffered liquid collagen (pH 7.4, 1.63 mg/ml collagen type I; Flow, McLean, Va.) containing minimum essential Eagle's medium (Sigma-Aldrich, Taufkirchen, Germany) as well as the investigated substances. This mixture was filled into self-constructed migration chambers [26], and allowed to polymerize for 30 min at 37 °C. The migration chamber consists of a normal microscopic glass slide at the base. On three sides, wax walls (vaseline and paraffin in a 1:1 mixture) were applied and a cover slip was mounted on top, resulting in a chamber of 10 mm × 10 mm × 0.5 mm. After polymerization of the collagen lattice, the chambers were sealed with the wax mixture on the fourth side, the migration of the cells was then recorded for 1 h (leukocytes) or 12 h (tumor cells). For analysis of the migration of tumor cells on two-dimensional surfaces, the cells were directly placed on a glass slide. Subsequently, chambers containing the cells were constructed as described above.

To stimulate migratory activity, we used for each cell type the most potent inducers of migration known. These were the stromal cell-derived factor-1 (SDF-1, 1 µg/ml; Biotrend Chemikalien, Cologne, Germany) for CTLs, formyl-methionyl-leucyl-phenylalanine (fMLP, 10 nM; Sigma-Aldrich) for neutrophil granulocytes, and norepinephrine (10 µM; Sigma-Aldrich) for tumor cells [27]. The MLCK inhibitor ML-7 (Calbiochem-Merck, Bad Soden, Germany) was used at 50 µM. The ROCK inhibitor Y-27632 (Calbiochem-Merck) was used at 10 µM. Blebbistatin (Sigma) was used at 100 µM. None of the inhibitors influenced the viability of the cells at these concentrations, as analyzed by propidium iodide staining and flow cytometry.

After recording, the migration paths of 30 randomly selected cells were digitized in 1-min intervals (leukocytes) or 15-min intervals (tumor cells) as previously described [25, 26]. The migratory activity was calculated for each step as the portion of the cells (in percent), which was locomotory active. All migratory activities shown in the figures are mean values of three independent experiments (for the leukocyte experiments, the cells of three different blood donors were used); in the text we provide the mean values and standard deviations (SD) at the steady-state level of the observation period. The velocity (μm/h) was calculated as the average of the mean single-cell velocities at the periods of actual movement (excluding pauses). Breaks are simply defined as periods in which the cells were not motile. The mean break time was calculated as the average of the duration of pauses per hour (minutes breaktime per hour) of each single cell. Statistical significance of changes was calculated using Student's t test.

Immunoblotting

The expression of myosin was analyzed by immunoblotting. Cells were lysed in Laemmli sample buffer (10 min at 95 °C), and lysates of 5×10^4 tumor cells or 1×10^6 leukocytes were applied to gel electrophoresis according to Laemmli [28]. The proteins were transferred to an Immobilion-P membrane (Millipore, Bedford, Mass.) and immunoblotting was performed as described previously [29]. For the detection of myosin, a mouse anti-myosin antibody (clone 2F12.A9; Coulter-Immunotech, Marseille, France) and a secondary horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Biosciences, Freiburg, Germany) were used.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from the leukocytes and tumor cells using the NucleoSpin RNA II Kit (Machery&Nagel, Dueren, Germany), and transcribed into cDNA in a reverse transcriptase reaction using oligo(dT) (Promega, Mannheim, Germany) and M-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany). The resulting cDNA was amplified using a PCR profile consisting of a 3-min denaturation at 94 °C and 35 cycles of 94 °C for 60 s and 50–60 °C for 60 s depending on the primer sequence, followed by a 60 s (90 s for MYH9 and MYO7A) elongation step at 72 °C. Myosin isotype-specific primer sequences were: 5′-TGG CTT GGG GAT TAA ACT ACC-3′ and 5′-CCT TTC CAA GCC ACA TGT TT-3′ for the brush border myosin-I (MYO1A); 5′-GAA ACA ATT TGA TTC CAA AGT-3′ and 5′-GAT TGG TAC AGG GTG CC-3′ for myosin-Iα (myr1, MYO1B); 5′-TGT GTG AGG TCA CCA CGT GT-3′ and 5′-CCT GCT CAG AAT CTG ATA CC-3′ for non-muscle myosin-IIa (MYH9) [30]; 5′-AGA CTT AGG TAC AAT TGC TCC CC-3′ and 5′-GAG AGA CAA GTG CAG TTT AGA GTC C-3′ for non-muscle myosin-IIb (MYH10); 5′-CCT GTT TAG TGC AGA GGA GG-3′ and 5′-AAA ATG TGT CTT CAG CTG GC-5′ for myosin-Va (MYO5A); 5′-TGT CAG CAT AAC TCC TTG ATG T-3′ and 5′-AGC TTT GTA ATT CAG AAA TCC TTG-3′ for myosin-VI (MYO6); 5′-TGA GAA TGT GCC GTT AGG-3′ and 5′-CCA GGG GAA AAG AAG ATG-3′ for myosin-VIIa (MYO7A) [31]. PCR products were detected by gel electrophoresis (1.5% agarose; Roth, Karlsruhe, Germany) and ethidium bromide staining.

Confocal laser-scanning microscopy

To analyze the intracellular distribution of actin, myosin, MLCK and ROCK-1, collagen lattices containing tumor cells and leukocytes were generated as described in the 'Cell migration assay and data analysis' section, with the difference that the mixtures were not filled into the migration chambers but were mounted on cover slips. The cover slips were placed in the wells of six-well plates. For the staining of tumor cells on two-dimensional surfaces, the cells were directly cultivated on cover slips. Immunofluorescent staining was then performed as described previously [32]. For the staining, we used the following antibodies: anti-MLCK goat polyclonal antibody (A-20), anti-ROCK-1 goat polyclonal antibody (K-18; both Santa Cruz Biotechnology, Heidelberg, Germany), and a rabbit anti-non-muscle myosin II antibody (Sigma-Aldrich). Filamentous actin was stained using Texas Red-conjugated phalloidin (Molecular Probes, Leiden, Netherlands). As secondary antibodies we used fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat (Zymed, San Francisco, Calif.) or goat anti-mouse antibodies (Beckman Coulter, Krefeld, Germany).

Confocal laser-scanning microscopy was performed using a TCS-4D microscope (Leica, Bensheim, Germany) as described previously [33]. Laser light of 488 and 568 nm was introduced. Both FITC and Texas Red fluorescences were detected in parallel, together with the transmission light in order to visualize the morphology of the cells. The confocal reflection contrast was used to visualize the collagen fibers. The cells were scanned in the z-axis by 1-μm intervals. Fluorescence and reflection contrast images were processed by an overlay of all z-scans.

Results

Myosin isotype expression

The conventional non-muscle motor protein myosin II is expressed in all cells we investigated, represented by a prominent band at 205 kDa (fig. 1A). Beside this, neutrophil granulocytes abundantly express a myosin protein of about 130/110 kDa, which is likely to be myosin I [34]. This protein is involved in phagocytic processes [35]. We then investigated the expression of those myosin isotypes, which were identified by immunoblotting (myosin I and

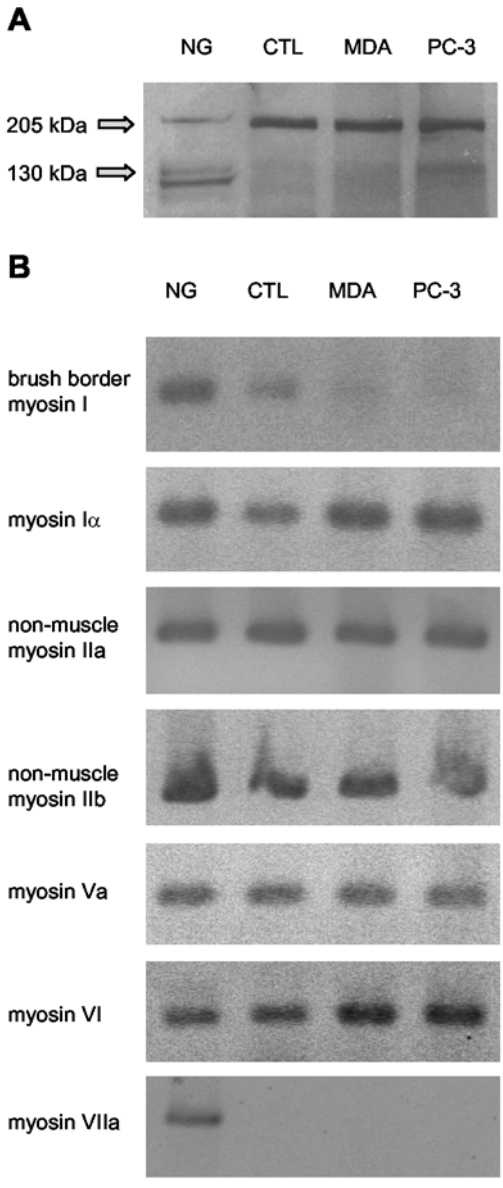

Figure 1. Expression of myosin isotypes in carcinoma cells and leukocytes. (*A*) Immunoblot analysis using a myosin antibody. (*B*) PCR analysis using isotype-specific primer sequences.

II), as well as the expression of other myosin isotypes with known function in the regulation of migration (myosin Va, VI, and VIIa) by RT-PCR (fig. 1B). Only the expression of myosin VIIa was restricted to neutrophil granulocytes, and the brush border myosin I was abundantly expressed in neutrophil granulocytes, whereas the other investigated cells revealed a weak expression of the RNA for the brush border myosin I. Since myosin VIIa is a very large protein of 2215 amino acids [36], the PCR analysis supports the conclusion drawn above that the extra band in neutrophil granulocytes is due to brush border myosin I expression.

Regulation of myosin by MLCK and ROCK

As described recently by the group of Nancy Hogg [21], both kinases that phosphorylate myosin – MLCK and ROCK – regulate the attachment and detachment of T lymphocytes on an ICAM-1-coated surface and are thus supposed to be involved in the regulation of migratory activity of these cells. Accordingly, at the same concentration range that was used by Hogg and colleagues [21], we found that the inhibition of ROCK by the specific inhibitor Y-27632 (10 μM) reduced the matrix-induced, spontaneous migration from 8.3±9.8% to 3.4±3.5% locomoting cells and led to a significant ($p = 0.015$) downregulation of the migration induced by the chemokine SDF-1 from 76.7±19.8% to 14.6±15.9% locomoting cells (fig. 2A). Likewise, inhibition of MLCK with the specific inhibitor ML-7 (50 μM) led to a downregulation of the matrix-induced, spontaneous migration from 14.5±12.7% to 1.0±1.4% locomoting cells and to a significant ($p < 0.001$) downregulation of the SDF-1-induced migration from 78.7±5.5% to 3.2±5.1% locomoting cells (fig. 2C). Among the myosin isotypes expressed in non-muscle cells, the type II myosins are the conventional motor proteins. We used blebbistatin at a concentration (100 μM) which has been shown to elicit maximal inhibitory effects: this concentration inhibited almost 90% of the maximal ATPase activity of human non-muscle myosin IIa and b [37], and reduced the filter transmigration of pancreatic adenocarcinoma cells in a Boyden chamber assay by 80% and more [38]. The spontaneous migration was completely inhibited by treatment of the CTLs with the non-muscle myosin II inhibitor blebbistatin (from 7.4±1.6% to 0.7±0.1% locomoting cells; $p = 0.02$) and after 1 h, the SDF-1-induced migration was reduced to the spontaneous activity (from 81.6±9.5% to finally 7.7±13.5% locomoting cells; $p = 0.01$; fig. 2E).

In contrast to CTLs, inhibition of ROCK with the same concentrations of Y-27632 (10 μM) only marginally affected the migration of neutrophil granulocytes (fig. 2B): fMLP-induced migration was only slightly reduced from 63.6±7.9% to 56.2±3.1% locomoting cells, and the spontaneous, matrix-induced migration remained almost unchanged (from 9.8±4.9% to 7.2±3.8% locomoting cells). Similar to CTLs, inhibition of the MLCK in neutrophil granulocytes strongly inhibited the fMLP-induced migratory activity from 61.2±6.9% to 34.8±23.8% locomoting cells (fig. 2D). However, this reduction did not reach statistical significance ($p = 0.19$) because of a high SD in the ML-7 treated population. Likewise, the spontaneous migration of neutrophil granulocytes was reduced by ML-7 (from 10.7±6.4% to 4.2±3.5% locomoting cells; fig. 2D). Similar to CTLs, in the neutrophil granulocytes, inhibition of non-muscle myosin II activity by blebbistatin completely prevented the spontaneous migration (from 9.8±6.7% to 2.8±1.1% locomoting cells) and reduced the fMLP-induced migratory activity down to the sponta-

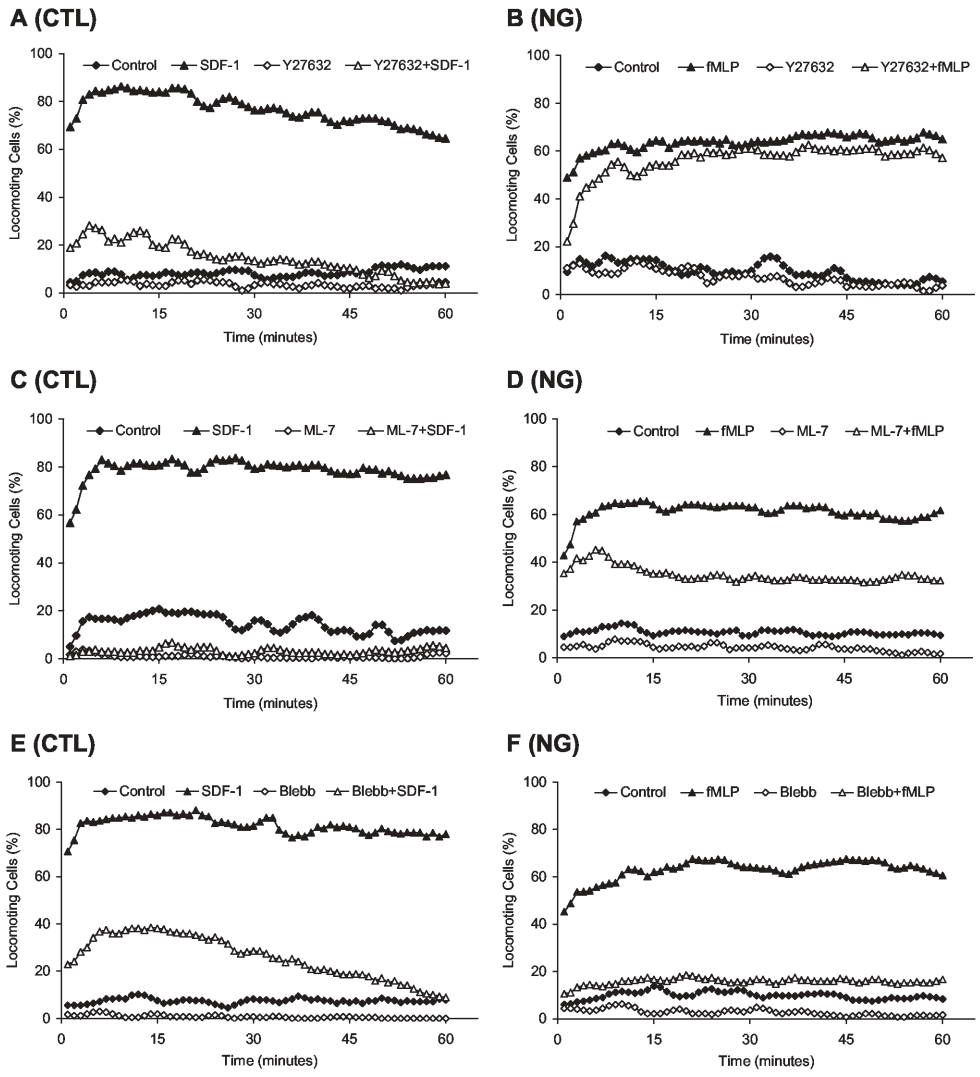

Figure 2. Effects of MLC phosphorylation inhibitors and of the non-muscle myosin II inhibitor blebbistatin on the migration of CTLs (*A*, *C*, *E*) and neutrophil granulocytes (NG) (*B*, *D*, *F*). In *A, B*, ROCK was inhibited using 10 μM Y-27632. *C*, *D* the MLCK was inhibited using 50 μM ML-7. *E*, *F* non-muscle myosin II activity was inhibited using 100 μM blebbistatin.

neous level (from 62.8±27.5% to 15.8±19.8% locomoting cells; fig. 2F). In summary, these results underscore the important role of non-muscle myosin II in leukocyte migration.

In contrast to the leukocytes, both MLC inhibitors (ML-7 and Y-27632) only partially reduced the migration of the tumor cells (fig. 3). Inhibition of ROCK by Y-27632 only slightly reduced the spontaneous migration of the MDA cells from 46.1±17.1% to 34.0±5.3% locomoting cells, but significantly ($p = 0.005$) reduced the norepinephrine-induced migration from 59.2±3.2% to 38.3±4.7% locomoting cells (fig. 3A). Likewise, inhibition of the MLCK by ML-7 reduced the spontaneous migration of the MDA cells from 41.7±9.5% to 36.0±12.8% locomoting cells, but the norepinephrine-induced migration from 58.6±10.2% down to 41.4±3.2% locomoting cells (fig. 3C). Thus, the norepinephrine-induced migration of MDA cells was reduced to spontaneous (control) levels, but the spontaneous migratory activity was only marginally affected. The use of the two inhibitors in combination did not further reduce the migratory activity but led to the same effect as was observed for each inhibitor alone (data not shown).

Although the overall migration activity of cells of the prostate carcinoma cell line PC-3 was much lower than of the MDA cells, we obtained similar results with the inhibitors ML-7 and Y-27632 (fig. 3B, D). An inhibition of ROCK in PC-3 cells reduced the spontaneous migration from 19.2±2.5% to 13.0±2.7% locomoting cells as well as the norepinephrine-induced migration from 27.1±7.6% to 12.2±1.7% locomoting cells (fig. 3B). Inhibition of MLCK did not influence the spontaneous

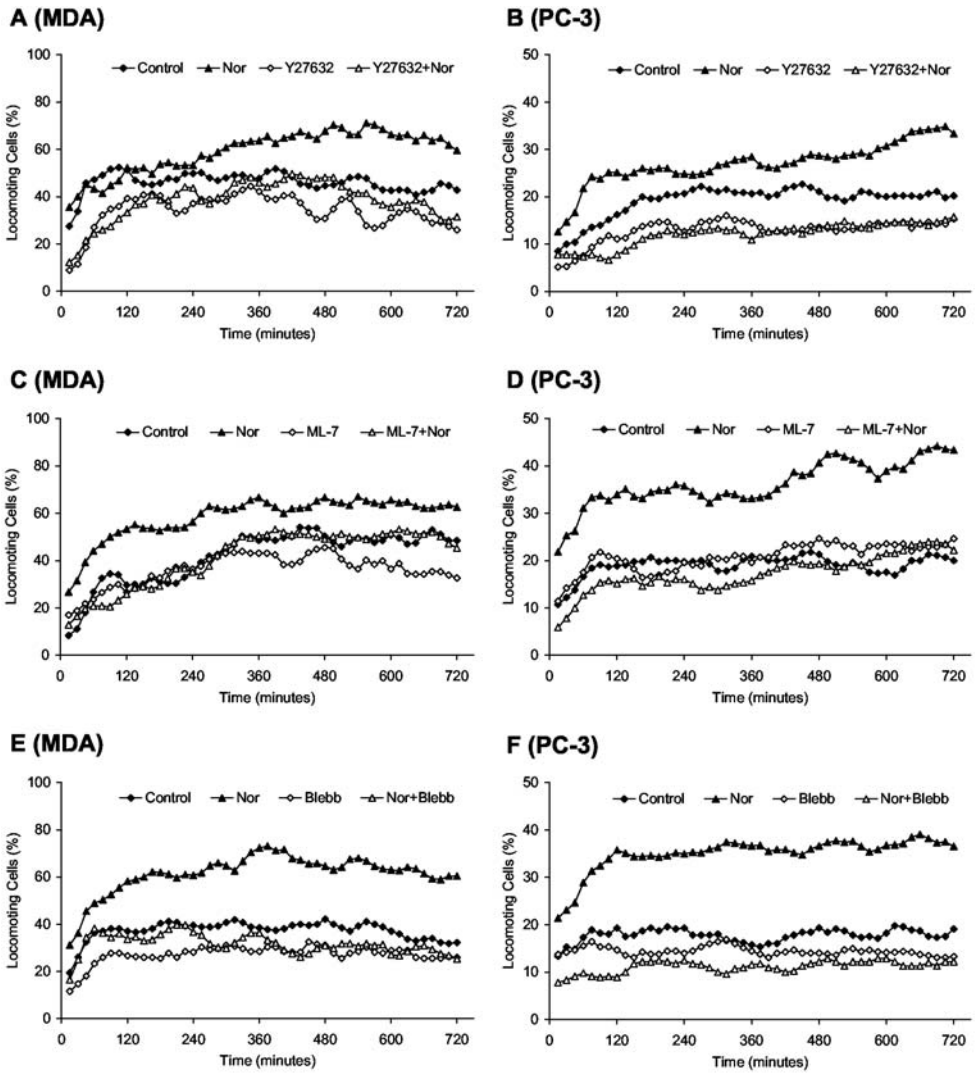

Figure 3. Effects of MLC phosphorylation inhibitors and of the non-muscle myosin II inhibitor blebbistatin on the migration of MDA-MB-468 breast carcinoma cells (MDA) (*A*, *C*, *E*) and PC-3 prostate carcinoma cells (*B*, *D*, *F*). Note: the scale of the y-axis is different in the graphs of MDA and PC-3 cells. *A*, *B* ROCK was inhibited using 10 μM Y-27632. *C*, *D* MLCK was inhibited using 50 μM ML-7. *E*, *F* non-muscle myosin II activity was inhibited using 100 μM blebbistatin.

migration (control = 19.0±1.9% locomoting cells vs 20.8±4.3% locomoting cells after ML-7 treatment), but significantly (p = 0.02) reduced the norepinephrine-induced migration from 36.3±6.4% locomoting cells to spontaneous migration activity, 17.3±3.0% locomoting cells (fig. 3D).

Since the inhibition of MLCK and ROCK did not completely impede the migratory activity of the tumor cells, we investigated whether the non-muscle myosin II is involved in the spontaneous or norepinephrine-induced migration of these cells. In accordance with our results on the inhibition of the MLC phosphorylating kinases, blebbistatin negated the promigratory effect of norepinephrine in both tumor cell lines (from 61.5±8.6% to 31.7±5.0% locomoting cells in MDA cells, p = 0.01, fig. 3E; and from 35.0±8.2% to 11.1±3.8% locomoting cells in PC-3 cells, p = 0.02, fig. 3F), whereas the spontaneous migration was only partially influenced (MDA: control = 37.3±10.9% and blebbistatin = 27.2±0.9% locomoting cells, fig. 3E: PC-3: control = 17.8±1.2% and blebbistatin = 14.4±10.5% locomoting cells, fig. 3F).

Further analysis of the migration behavior showed that the velocity of migration increased in both cell lines (MDA: from 12.6±3.7 μm/h to 26.9±2.0 μm/h; PC-3: from 12.1±0.1 μm/h to 19.5±8.1 μm/h), which was, however, only significant in MDA cells (fig. 4A, B). Likewise, the duration of breaks per hour performed by the cells between phases of migratory activity was reduced by norepinephrine treatment (MDA: from 43.2±3.2 min/h to 22.9±5.7 min/h; PC-3: from 44.6±0.8 min/h to 35.7±10.7 min/h; fig. 4C, D). Treatment with blebbistatin alone had no effect on the migratory behavior of MDA

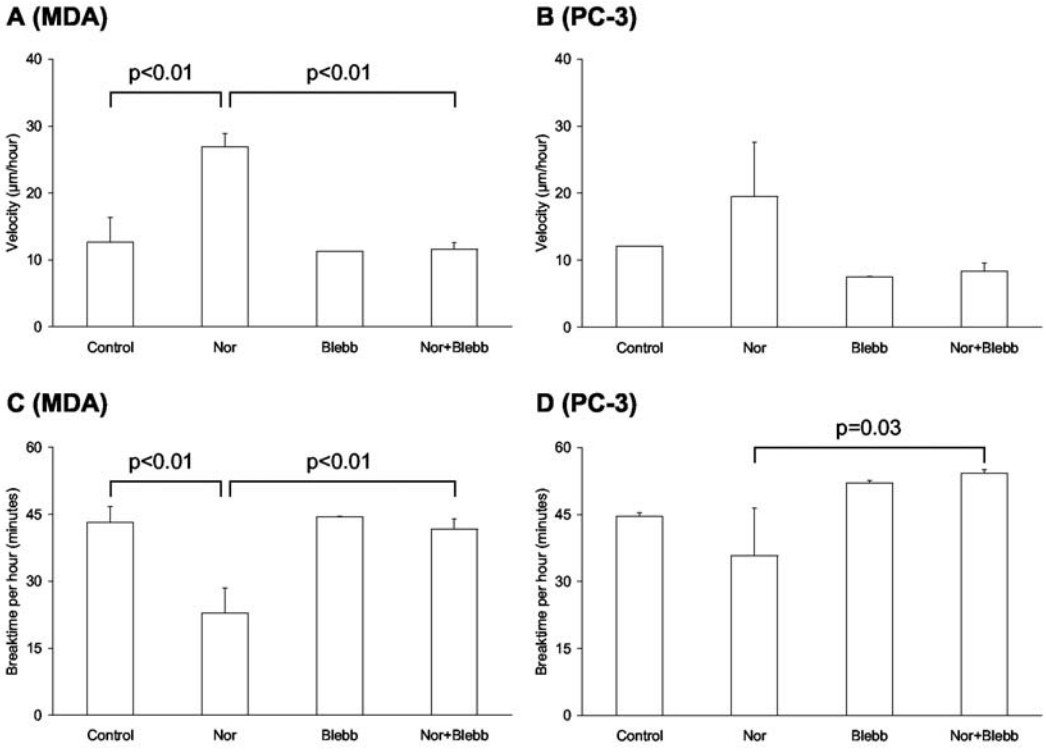

Figure 4. Analysis of the velocity (µm/h; *A*, *C*) and break duration (min/h; *B*, *D*) of the migration of MDA-MB-468 breast carcinoma cells (MDA) (*A*, *C*) and PC-3 prostate carcinoma cells (*B*, *D*) deduced from the migration experiments shown in figure 3. Columns show the mean values and SD of three independent experiments (in total 90 individual cells were analyzed per sample).

cells (velocity 11.3±0.1 µm/h and breaktime 44.5±0.1 min/h; fig. 4A, C), and only slightly affected the migration of PC-3 cells (velocity 7.5±0.1 µm/h and breaktime 52.0±0.5 min/h; fig. 4B, D). However, according to the reduction of migratory activity, blebbistatin completely abolished the norepinephrine effect (MDA: velocity 11.6±1.0 µm/h and breaktime 41.7±2.2 min/h; PC-3: velocity 8.3±1.2 µm/h and breaktime 54.2±0.9 min/h, fig. 4).

Intracellular distribution of actin, myosin, MLCK and ROCK-1 in tumor cells

Immunostaining of non-muscle myosin II in tumor cells revealed a cortical localization in migrating tumor cells (figs. 5A, 6A, second column), which resembles, but is however not identical to the distribution of filamentous actin (figs. 5A, 6A, third column). The migrating tumor cells interact tightly with the surrounding matrix, which leads to a remodeling of the collagen network (e.g., bundling of fibers). At certain matrix contact sites of the cells, high cellular concentrations of non-muscle myosin II were co-localized with high concentrations of filamentous actin (white arrowheads), whereas at some other matrix contact sites, only higher amounts of filamentous actin, but not of myosin were found (red arrowheads). We made similar observations for the intracellular distribution of MLCK (figs. 5B, 6B) and ROCK-1 (figs. 5C, 6C). Furthermore, in the three-dimensional collagen system, the migrating tumor cells did not develop any stress fibers, a phenomenon which is frequently observed in two-dimensional systems (fig. 7). On the glass surface, the cells spread but did not develop any migratory activity (data not shown).

Discussion

Migration is a cell function which is tightly regulated by extracellular signal substances. These migration-inducing signals are either provided by the surrounding (collagen) matrix via integrin receptors [39, 40], or by soluble signal substances. One large family of such substances are the ligands to serpentine receptors or G protein-coupled receptors. This family mainly consists of the chemokines, which are the most important regulators for leukocyte migration and homing, and of the neurotransmitters, which are also regulators for both the migration of leukocytes and tumor cells [41]. On the intracellular side, serpentine receptors induce signal transduction pathways, which lead to an increase in cytosolic calcium and the activation of protein kinase A and C (PKA and PKC) [42, 43] as key regulatory events for the migration of both leukocytes and tumor cells (fig. 8): serpentine receptors are coupled on the intracellular side to

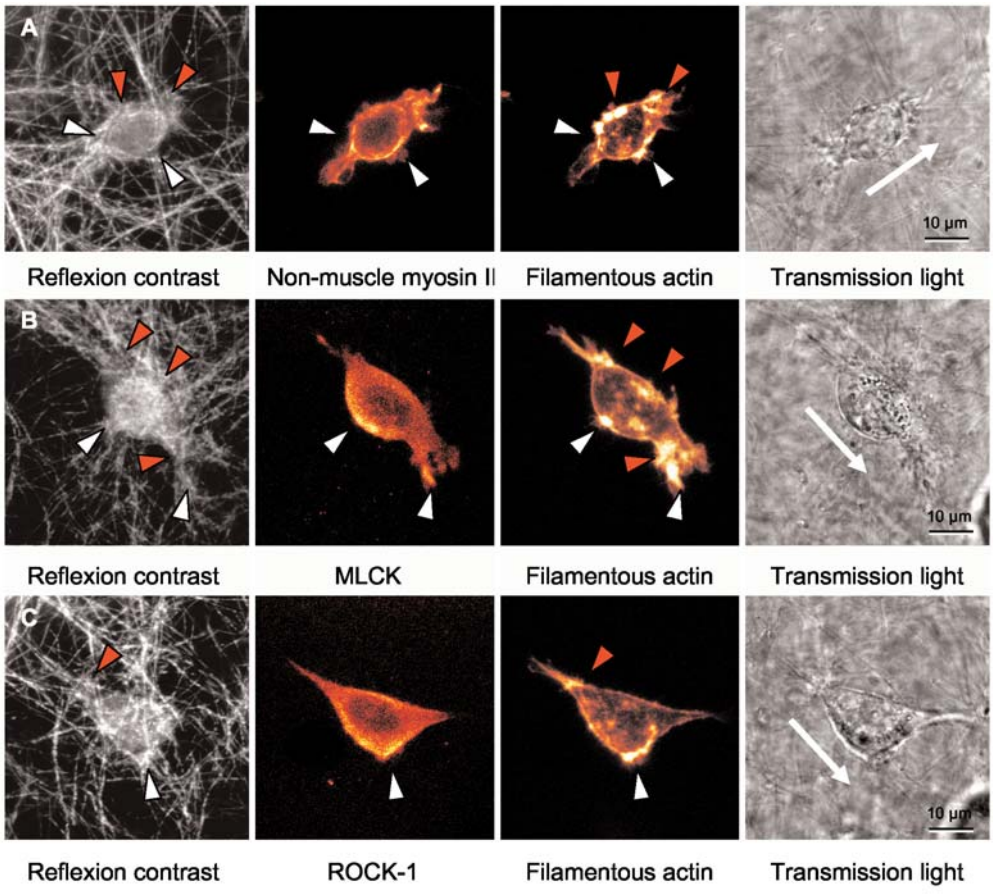

Figure 5. Intracellular distribution of non-muscle myosin II, MLCK, and ROCK in comparison to filamentous actin in migrating MDA-MB-468 breast carcinoma cells. White arrowheads depict matrix contact sites with a co-localization of filamentous actin with non-muscle myosin II (*A*), MLCK (*B*), and ROCK-1 (*C*). Red arrowheads depict matrix contact sites with filamentous actin alone.

heterotrimeric G proteins, which in turn activate two signaling pathways of importance to cell migration. Upon activation, G proteins disengage into a GTP-bound α subunit and a $\beta\gamma$ subunit. The adenylyl cyclase is a key target molecule of the α subunit. Depending on the receptor type, stimulatory (Gs) α proteins or inhibitory (Gi) α proteins are activated, which increase or inhibit, respectively, the enzymatic activity of the adenylyl cyclase and thereby regulate the production of cAMP. Protein kinase A (PKA) is activated by cAMP. PKA regulates the sequestration of cytosolic calcium via phospholamban (PLB) and the sarcoplasmatic/endoplasmatic calcium ATPase (SERCA). The second pathway is activated by the $\beta\gamma$ subunit of the G proteins. This part of the G proteins activates G protein-coupled receptor kinases (GRKs), which engage src protein tyrosine kinases (srcPTKs) via β-arrestin. A key target substrate of the srcPTKs is phospholipase C (PLC)γ. PLCγ catalyzes the breakdown of phosphatidylinositolbisphosphate (PIP_2) to the metabolites diacylglycerol (DAG), an activator of PKCα, and inositol-1,4,5-phosphate (IP_3), a second messenger which opens intracellular calcium channels.

But these signaling events regulate the myosin activity: calcium is the activator for CaM/MLCK, and the GTPase activity of Rho, which is in turn the activator for ROCK, is under the dual control of PKA and PKC [44]. PKC activates Rho via the activation of guanine exchange factors [45], whereas in opposition, PKA inhibits Rho via the activation of guanine dissociation inhibitors [46, 47]. Accordingly, we have shown that the activation of PKC induces migration of tumor cells, while activation of PKA functions as an inhibitor [32, 48]. An alternative pathway for the activation of MLCK by the mitogen-activated protein kinase (MAPK) has been described by Klemke and co-workers [49]. This pathway is initiated by integrins or cytokine receptors such as epidermal growth factor receptor.

Smith et al. [21] have recently deduced from their results that in T lymphocytes, both activators of myosin, MLCK and ROCK-1, are important for migration with regard to the integrin-mediated attachment to and detachment from an ICAM-1-coated surface. They reported that MLCK is located at the leading edge of migrating T lymphocytes, and ROCK-1 is located in the trailing uropod. Accord-

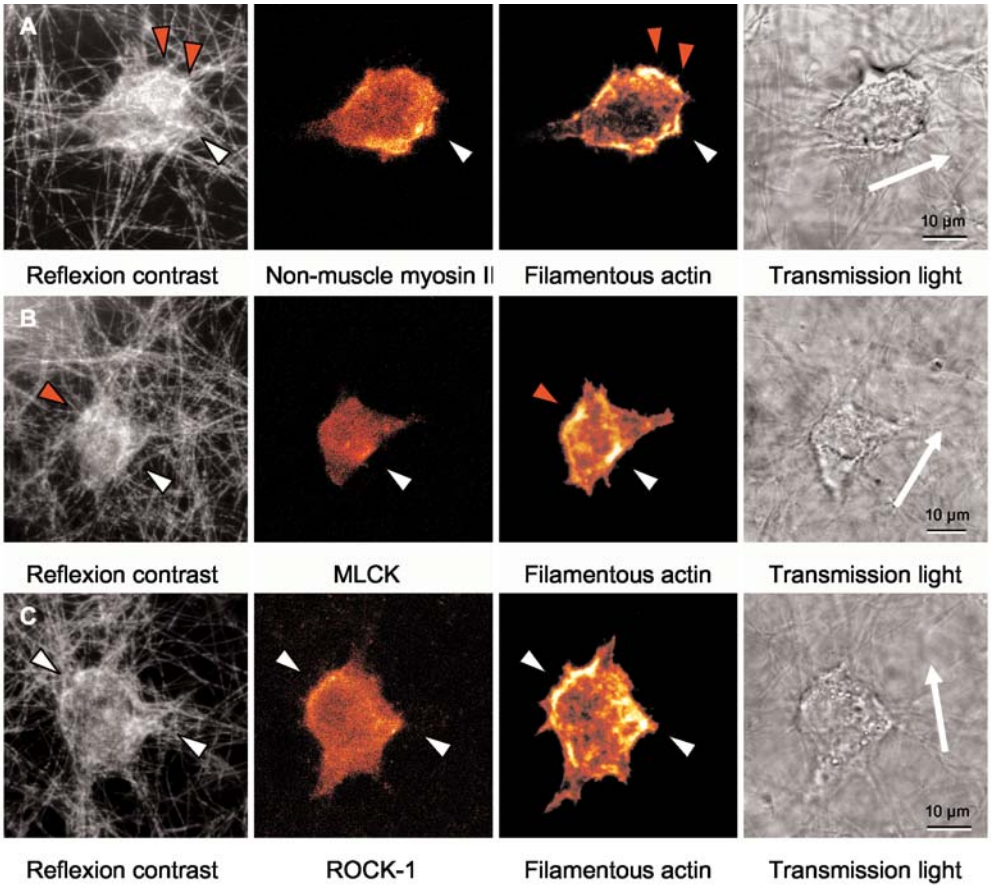

Figure 6. Intracellular distribution of non-muscle myosin II, MLCK, and ROCK in comparison to filamentous actin in migrating PC-3 prostate carcinoma cells. White arrowheads depict matrix-contact sites with a colocalization of filamentous actin with non-muscle myosin II (*A*), MLCK (*B*), and ROCK-1 (*C*). Red arrowheads depict matrix contact sites with filamentous actin alone.

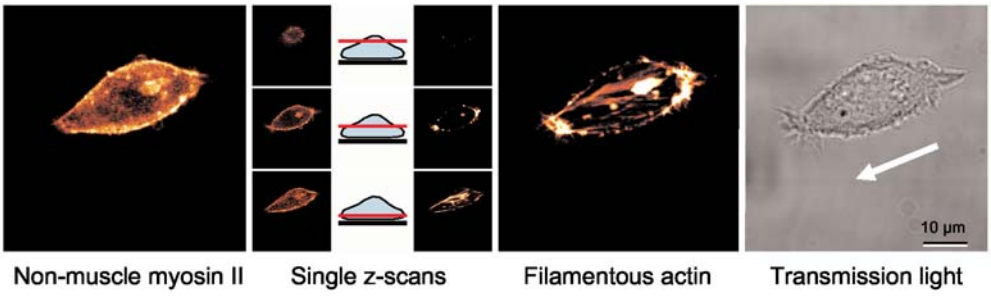

Figure 7. Intracellular distribution of non-muscle myosin II and filamentous actin in breast carcinoma cells cultivated on a glass surface. Single z-scans illustrate the spatial distribution of myosin and actin; the red lines indicate the level within the cell (gray).

ingly, we found that both kinases are essential for the migration of CTLs in a three-dimensional collagen lattice. In neutrophil granulocytes, an inhibition of the calcium-dependent phosphorylation of the MLC led to a strong reduction of migratory activity, while the inhibition of Rho-dependent pathways had almost no effect. These findings are in accordance with results of Eddy and co-workers [50], who showed that inhibition of MLCK led to an impaired uropod retraction. Furthermore, buffering of intracellular calcium transients led to similar effects [50]. We have shown previously that an increase in cytosolic calcium occurs at the leading edge of a migrating neutrophil granulocyte [51]. Regarding the results of Eddy et al. [50], the increase in cytosolic calcium therefore occurs at that cellular region where myosin II is located.

While the aforementioned results give us a relatively clear overview of the involvement of myosin in leukocyte migration and a broad basis for further research, much

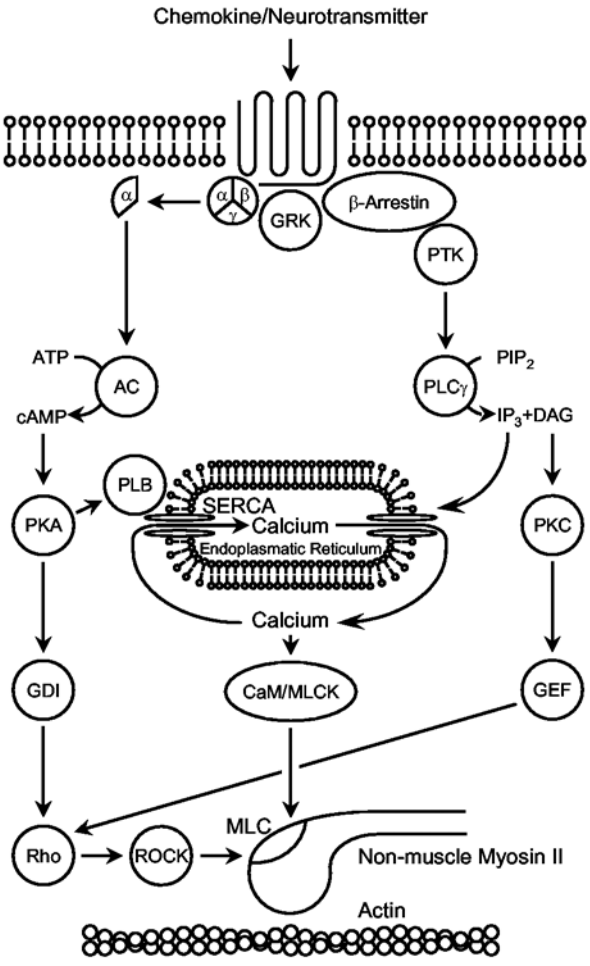

Figure 8. Schema of chemokine/neurotransmitter-induced signal transduction pathways regulating cell migration.

less is known about how myosin is regulated in migrating tumor cells and where it is localized. Our results show that, in contrast to leukocytes, neither the inhibition of MLCK nor of ROCK alone or in combination with each other completely inhibited tumor cell migration. The neurotransmitter-induced migration was completely prevented, but the spontaneous migratory activity was only slightly impaired. Inhibition of non-muscle myosin II activity with the specific inhibitor blebbistatin confirmed these results. We conclude that non-muscle myosin II is activated by the above-described pathways after engagement of serpentine receptors (i.e., beta2-adrenoceptors [26, 43]) by norepinephrine binding, and does not contribute to the spontaneous migration, which is induced by the matrix via integrin receptors. This spontaneous migration might either be independent of non-muscle myosin II in the generation of motor forces, e.g. via exclusively actin-based migration mechanisms, or other myosin isotypes are involved in this type of migration, such as myosin VI, whose role in ovarian cancer dissemination has been investigated recently [13]. The differences in the molecular signaling between spontaneous migration and norepinephrine-induced migration are furthermore reflected in the migratory dynamics: we have described previously that tumor cells significantly increase the velocity of migration after norepinephrine treatment and concomitantly significantly reduce the length of migratory breaks [52]. Here, we showed that these changes of the migratory dynamics are based on the activation of non-muscle myosin II (fig. 4). This leads to the conclusion that non-muscle myosin II is essential for both the spontaneous and serpentine receptor-induced migration of leukocytes, whereas in tumor cells, non-muscle myosin II is involved only in the serpentine receptor-induced migration and not in the spontaneous locomotion.

Not only the regulation, but also the intracellular distribution of non-muscle myosin II differs in tumor cells and leukocytes. In tumor cells, MLCK and ROCK-1 do not show the distinct, exclusive distribution of MLCK at the leading front and ROCK-1 at the rear end, as was observed in T lymphocytes [21]. Furthermore, filamentous actin and non-muscle myosin II did not form stress fibers, which has been shown for tumor cells on two-dimensional surfaces [53]. In a three-dimensional environment, as used in this study, filamentous actin and non-muscle myosin II in tumor cells are predominantly located at matrix contact sites, i.e., focal adhesion contacts, where they are co-localized with MLCK and ROCK-1. Focal adhesion contacts are an exclusive structure of migrating tumor cells and are not developed by T lymphocytes in three-dimensional matrices [33]. The differences in the localization support the functional findings and the hypothesis that the involvement of myosin in the regulation of migration is different in leukocytes and tumor cells. However, the expression profile of myosin isotypes is almost the same in these cell types. The myosin II isotype is ubiquitously expressed and has accordingly been found in all cell types investigated in this study. Besides myosin II, myosin Va and VIIa are expressed in myeloid stem cells [54]. Congruently, all these myosin isotypes are present in neutrophil granulocytes (fig. 1B), whereas myosin VIIa is not expressed in T lymphocytes or tumor cells.

In summary, the fast migration of leukocytes essentially depends on the activity of non-muscle myosin II. In slow-moving tumor cells, only the norepinephrine-induced migration involves the activity of non-muscle myosin II. Therefore, the expression of proteins which regulate migratory activity and produce motile forces is similar in leukocytes and tumor cells, but the concert of these proteins is distinct.

Acknowledgements. This work was supported by the Fritz Bender Foundation, Munich, and the Deutsche Krebshilfe, Bonn, Germany. We thank B. Mainusch for excellent technical assistance, as well as D. Palm and F. Kreisz for assistance with the migration experiments.

1 Wehrle-Haller B. and Imhof B. A. (2002) The inner lives of focal adhesions. Trends Cell Biol. **12:** 382–389
2 DeMali K. A., Barlow C. A. and Burridge K. (2002) Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. J. Cell Biol. **159:** 881–891
3 Entschladen F. and Zanker K. S. (2000) Locomotion of tumor cells: a molecular comparison to migrating pre- and postmitotic leukocytes. J. Cancer Res. Clin. Oncol. **126:** 671–681
4 Horwitz A. R. and Parsons J. T. (1999) Cell migration – movin' on. Science **286:** 1102–1103
5 Ishii Y. and Yanagida T. (2002) A new view concerning an actomyosin motor. Cell. Mol. Life Sci. **59:** 1767–1770
6 Berg J. S., Powell B. C. and Cheney R. E. (2001) A millennial myosin census. Mol. Biol. Cell **12:** 780–794
7 Kolega J. (2003) Asymmetric distribution of myosin IIB in migrating endothelial cells is regulated by a rho-dependent kinase and contributes to tail retraction. Mol. Biol. Cell **14:** 4745–4757
8 Al-Haddad A., Shonn M. A., Redlich B., Blocker A., Burkhardt J. K., Yu H. et al. (2001) Myosin Va bound to phagosomes binds to F-actin and delays microtubule-dependent motility. Mol. Biol. Cell **12:** 2742–2755
9 Provance D. W. and Mercer J. A. (1999) Myosin-V: head to tail. Cell. Mol. Life Sci. **56:** 233–242
10 Redowicz M. J. (2002) Myosins and pathology: genetics and biology. Acta Biochim. Pol. **49:** 789–804
11 Geisbrecht E. R. and Montell D. J. (2002) Myosin VI is required for E-cadherin-mediated border cell migration. Nat. Cell Biol. **4:** 616–620
12 Buss F., Luzio J. P. and Kendrick-Jones J. (2002) Myosin VI, an actin motor for membrane traffic and cell migration. Traffic **3:** 851–858
13 Yoshida H., Cheng W., Hung J., Montell D., Geisbrecht E., Rosen D. et al. (2004) Lessons from border cell migration in the *Drosophila* ovary: a role for myosin VI in dissemination of human ovarian cancer. Proc. Natl. Acad. Sci. USA **101:** 8144–8149
14 Tuxworth R. I., Weber I., Wessels D., Addicks G. C., Soll D. R., Gerisch G. et al. (2001) A role for myosin VII in dynamic cell adhesion. Curr. Biol. **11:** 318–329
15 Van Dijk J., Knight A. E., Molloy J. E. and Chaussepied P. (2002) Characterization of three regulatory states of the striated muscle thin filament. J. Mol. Biol. **323:** 475–489
16 Stephens N. L. (2001) Airway smooth muscle. Lung **179:** 333–373
17 Pfitzer G. (2001) Invited review: regulation of myosin phosphorylation in smooth muscle. J. Appl. Physiol. **91:** 497–503
18 Riento K. and Ridley A. J. (2003) Rocks: multifunctional kinases in cell behaviour. Nat. Rev. Mol. Cell. Biol. **4:** 446–456
19 Yamashiro S., Totsukawa G., Yamakita Y., Sasaki Y., Madaule P., Ishizaki T. et al. (2003) Citron kinase, a Rho-dependent kinase, induces di-phosphorylation of regulatory light chain of myosin II. Mol. Biol. Cell **14:** 1745–1756
20 Wolf W. A., Chew T. L. and Chisholm R. L. (1999) Regulation of cytokinesis. Cell. Mol. Life Sci. **55:** 108–120
21 Smith A., Bracke M., Leitinger B., Porter J. C. and Hogg N. (2003) LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment. J. Cell Sci. **116:** 3123–3133
22 Yanase M., Ikeda H., Ogata I., Matsui A., Noiri E., Tomiya T. et al. (2003) Functional diversity between Rho-kinase- and MLCK-mediated cytoskeletal actions in a myofibroblast-like hepatic stellate cell line. Biochem. Biophys. Res. Commun. **305:** 223–228
23 Kaneko K., Satoh K., Masamune A., Satoh A. and Shimosegawa T. (2002) Myosin light chain kinase inhibitors can block invasion and adhesion of human pancreatic cancer cell lines. Pancreas **24:** 34–41
24 Webb D. J., Donais K., Whitmore L. A., Thomas S. M., Turner C. E., Parsons J. T. et al. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat. Cell Biol. **6:** 154–161
25 Entschladen F., Gunzer M., Scheuffele C. M., Niggemann B. and Zanker K. S. (2000) T lymphocytes and neutrophil granulocytes differ in regulatory signaling and migratory dynamics with regard to spontaneous locomotion and chemotaxis. Cell. Immunol. **199:** 104–114
26 Drell T. L., Joseph J., Lang K., Niggemann B., Zaenker K. S. and Entschladen F. (2003) Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. Breast Cancer Res. Treat. **80:** 63–70
27 Joseph J., Niggemann B., Zaenker K. S. and Entschladen F. (2004) Anandamide is an endogenous inhibitor for the migration of tumor cells and T lymphocytes. Cancer Immunol. Immunother. **53:** 723–728
28 Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
29 Entschladen F., Niggemann B., Zanker K. S. and Friedl P. (1997) Differential requirement of protein tyrosine kinases and protein kinase C in the regulation of T cell locomotion in three-dimensional collagen matrices. J. Immunol. **159:** 3203–3210
30 Linz-McGillem L. A. and Alliegro M. C. (2003) Myosin II in retinal pigmented epithelial cells: evidence for an association with membranous vesicles. Exp. Eye Res. **76:** 543–552
31 Kelley P. M., Weston M. D., Chen Z. Y., Orten D. J., Hasson T., Overbeck L. D. et al. (1997) The genomic structure of the gene defective in Usher syndrome type Ib (MYO7A). Genomics **40:** 73–79
32 Masur K., Lang K., Niggemann B., Zanker K. S. and Entschladen F. (2001) High PKC alpha and low E-cadherin expression contribute to high migratory activity of colon carcinoma cells. Mol. Biol. Cell **12:** 1973–1982
33 Friedl P., Entschladen F., Conrad C., Niggemann B. and Zanker K. S. (1998) CD4+ T lymphocytes migrating in three-dimensional collagen lattices lack focal adhesions and utilize beta1 integrin-independent strategies for polarization, interaction with collagen fibers and locomotion. Eur. J. Immunol. **28:** 2331–2343
34 Balish M. F., Moeller E. F. 3rd and Coluccio L. M. (1999) Overlapping distribution of the 130- and 110-kDa myosin I isoforms on rat liver membranes. Arch. Biochem. Biophys. **370:** 285–-293
35 Allen L. H. and Aderem A. (1995) A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. J. Exp. Med. **182:** 829–840
36 Kussel-Andermann P., El-Amraoui A., Safieddine S., Nouaille S., Perfettini I., Lecuit M. et al. (2000) Vezatin, a novel transmembrane protein, bridges myosin VIIA to the cadherin-catenins complex. EMBO J. **19:** 6020–6029
37 Straight A. F., Cheung A., Limouze J., Chen I., Westwood N. J., Sellers J. R. et al. (2003) Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. Science **299:** 1743–1747
38 Duxbury M. S., Ashley S. W. and Whang E. E. (2004) Inhibition of pancreatic adenocarcinoma cellular invasiveness by blebbistatin: a novel myosin II inhibitor. Biochem. Biophys. Res. Commun. **313:** 992–997
39 Maaser K., Wolf K., Klein C. E., Niggemann B., Zanker K. S., Brocker E. B. et al. (1999) Functional hierarchy of simultaneously expressed adhesion receptors: integrin alpha2beta1 but not CD44 mediates MV3 melanoma cell migration and matrix reorganization within three-dimensional hyaluronan-containing collagen matrices. Mol. Biol. Cell **10:** 3067–3079
40 Volkov Y., Long A., McGrath S., Ni Eidhin D. and Kelleher D. (2001) Crucial importance of PKC-beta(I) in LFA-1-mediated locomotion of activated T cells. Nat Immunol **2:** 508–514

41 Entschladen F., Drell T. L., Lang K., Joseph J. and Zaenker K. S. (2004) Tumour cell migration, invasion and metastasis: navigation by neurotransmitters. Lancet Oncol. **5:** 254–258
42 Pierce K. L., Premont R. T. and Lefkowitz R. J. (2002) Seven-transmembrane receptors. Nat. Rev. Mol. Cell Biol. **3:** 639–650
43 Lang K., Drell T. L., Lindecke A., Niggemann B., Kaltschmidt C., Zaenker K. S. et al. (2004) Induction of a metastatogenic tumor cell type by neurotransmitters and its pharmacological inhibition by established drugs. Int. J. Cancer **112:** 231–238
44 Laudanna C., Campbell J. J. and Butcher E. C. (1997) Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. J. Biol. Chem. **272:** 24141–24144
45 Sagi S. A., Seasholtz T. M., Kobiashvili M., Wilson B. A., Toksoz D. and Brown J. H. (2001) Physical and functional interactions of Galphaq with Rho and its exchange factors. J. Biol. Chem. **276:** 15445–15452
46 Ellerbroek S. M., Wennerberg K. and Burridge K. (2003) Serine phosphorylation negatively regulates RhoA in vivo. J. Biol. Chem. **278:** 19023–19031
47 Somlyo A. P. and Somlyo A. V. (2003) Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. **83:** 1325–1358
48 Joseph J., Niggemann B., Zaenker K. S. and Entschladen F. (2002) The neurotransmitter gamma-aminobutyric acid is an inhibitory regulator for the migration of SW 480 colon carcinoma cells. Cancer Res. **62:** 6467–6469
49 Klemke R. L., Cai S., Giannini A. L., Gallagher P. J., Lanerolle P. de and Cheresh D. A. (1997) Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol. **137:** 481–492
50 Eddy R. J., Pierini L. M., Matsumura F. and Maxfield F. R. (2000) Ca2+-dependent myosin II activation is required for uropod retraction during neutrophil migration. J. Cell Sci. **113:** 1287–1298
51 Lang K., Hatt H., Niggemann B., Zaenker K. S. and Entschladen F. (2003) A novel function for chemokines: down-regulation of neutrophil migration. Scand. J. Immunol. **57:** 350–361
52 Niggemann B., Drell T. L., Joseph J., Weidt C., Lang K., Zaenker K. S. et al. (2004) Tumor cell locomotion: differential dynamics of spontaneous and induced migration in a 3D collagen matrix. Exp. Cell Res. **298:** 178–187
53 Bresnick A. R. (1999) Molecular mechanisms of nonmuscle myosin-II regulation. Curr. Opin. Cell Biol. **11:** 26–33
54 Zenzmaier C., Kollroser M., Gesslbauer B., Jandrositz A., Preisegger K. H. and Kungl A. J. (2003) Preliminary 2-D chromatographic investigation of the human stem cell proteome. Biochem. Biophys. Res. Commun. **310:** 483–490