Microtubule-disruption-induced and chemotactic-peptide-induced migration of human neutrophils: implications for differential sets of signalling pathways

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Summary

Neutrophil granulocytes rely on a functional actin network for directed migration. Microtubule disassembly does not impair receptor-linked chemotaxis, instead it induces development of polarity and chemokinesis in neutrophils concomitant with polarized distribution of α-actinin and F-actin. Cells stimulated with colchicine, which disassembles microtubules, migrate with a speed comparable to cells exposed to chemotactic peptide. We investigated signalling pathways involved in colchicine-induced neutrophil polarization and migration. Colchicine-induced development of polarity was insensitive to treatment with pertussis toxin, in contrast to chemotactic-peptide-induced shape changes, which were completely abolished by this treatment. Thus, colchicine does not appear to act via activating heterotrimeric G_i proteins. Colchicine does also not seem to act via phosphatidylinositol 3-kinase, as it failed to induce phosphorylation of its downstream target Akt and the potent phosphatidylinositol 3-kinase inhibitor wortmannin failed to inhibit colchicine-induced shape changes. By contrast, wortmannin significantly reduced chemotactic-peptide-induced shape changes. However, the Rho-kinase inhibitor Y-27632 (10 μM) inhibited colchicine-induced development of polarity by 95±3% (*n*=5) and chemokinesis by 76±9% (*n*=3), which suggests that the Rho-Rho-kinase pathway has a crucial role in polarity and migration. Indeed, treatment of cells with colchicine induced a significant increase in membrane-bound Rho-kinase II, which is indicative of activation of this protein. This membrane translocation could be prevented by taxol, which stabilizes microtubules. Colchicine also induced a marked increase in myosin light chain phosphorylation, which could be suppressed by Y-27632 and by taxol. In summary, we provide evidence that microtubule disassembly induces in neutrophils a selective activation of Rho-kinase, bypassing activation of heterotrimeric Gi proteins and phosphatidylinositol 3-kinase. This process is sufficient for induction of chemokinesis and mediates increased phosphorylation of myosin light chain and accumulation of F-actin and α-actinin in the leading edge.

Key words: Neutrophil, Microtubule, Migration, Rho, Rho-kinase, Phosphatidylinositol 3-kinase

Introduction

Directed migration of neutrophils is essential for the fulfillment of their physiological role, that is, defending the host against invading bacteria. Neutrophils develop polarity and migrate not only when exposed to a gradient of chemoattractant (chemotaxis) but also in uniform concentrations of stimulus (stimulated random migration, known as chemokinesis). Reversible actin polymerization is crucial for neutrophil development of polarity and migration (Chung et al., 2001). Less is known about the role of microtubules. The latter have been extensively studied in motile events in fibroblasts and growth cones. There, disassembly of microtubules attenuates polarity and migration (Vasiliev et al., 1970; Tanaka et al., 1995) and increases contractility and myosin light chain phosphorylation (Kolodney and Elson, 1995). In neutrophils, by contrast, an intact microtubule network does not appear to be required for migration. Microtubule-disrupting drugs such as colchicine, vinblastine or nocodazole even induce development of polarity and chemokinesis (Dziezanowski et al., 1980; Keller et al., 1984). Microtubule depolymerization does not impair chemotaxis induced by activated serum (Dzienzanowski et al., 1980) or by the chemotactic peptide f-Met-Leu-Phe (fMLP), and neutrophils exposed to colchicine still orient in a gradient of chemotactic peptide (Zigmond, 1977; Keller et al., 1984). Colchicine itself does not act as a chemoattractant. Interestingly, colchicine-induced motile responses can be abolished by cytochalasin B, indicating a role for actin filaments (Keller et al., 1984). A crosstalk between microtubules and actin filaments is further substantiated by the observation that colchicine treatment induces an increase in cyoskeleton-associated actin and α-actinin as well as enrichment of actin, α-actinin and tubulin in the front and myosin II at the tail of the polarized cells (Keller and Niggli, 1993). Microtubule depolymerization thus induces changes in the actin network that are required for the increased motility of neutrophils. One possible interpretation of these earlier

findings is based on interactions between the different filament systems, where the modification of one system affects the others [‘tensegrity’ (for details, see Ingber, 1993)]. Recent evidence obtained in fibroblasts suggests a different explanation. Enomoto showed that in 3T3 fibroblasts microtubule depolymerization by colcemid, colchicine or nocodazole induces formation of stress fibers and vinculin-containing focal adhesions (Enomoto, 1996). These effects are inhibited by cytochalasin D, which prevents addition of actin monomers to the barbed ends of actin filaments, and taxol, which stabilizes microtubules. Interestingly, these responses to microtubule depolymerization were inhibited by exoenzyme C3. This protein selectively ADP-ribosylates and inhibits the small GTP-binding protein Rho without affecting rac or cdc42. An inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase) did not suppress the responses (Enomoto, 1996; Liu et al., 1998).

Microtubule disruption thus may activate Rho, at least in 3T3 cells (Ren et al., 1999). Another small GTP-binding protein, Rac1, in contrast, is activated in those cells by microtubule growth, resulting in increased lamellipodial protrusions in fibroblasts (Waterman-Storer et al., 1999). Microtubule growth at the leading edge and shortening at the rear could thus locally activate Rho or Rac and result in local contraction or ruffling in migrating fibroblasts (Wittmann and Waterman-Storer, 2001). In contrast to the data obtained in neutrophils, microtubule disruption reduces polarization, ruffling and the speed of migration in fibroblasts (Wittmann and Waterman-Storer, 2001), which suggests that different mechanisms operate in the different cell types.

We describe here the signalling from microtubule disruption to migration in human neutrophils. We provide evidence for the activation in this process of Rho-kinase but not of heterotrimeric Gi proteins, the PI 3-kinase pathway or the p42/44 mitogen-activated protein kinase (MAPK). This differs from chemotactic peptide-induced migration where Gi proteins are activated as well as PI 3-kinase and the MAPK cascade in addition to Rho family proteins and their downstream targets.

Materials and Methods

Materials

Reagents and suppliers were N-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-norleucyl-L-tyrosyl-L-lysine (fNLPNTL), Bachem, Bubendorf, Switzerland; human serum albumin (HSA), ZLB Bioplasma AG, Bern, Switzerland; neutrophil isolation medium (NIM), Cardinal Associates, Santa Fe, NM, USA; OptiPrep, Axis-Shield PoC As, Oslo, Norway; dimethylsulfoxide (DMSO), enhanced chemiluminescence (ECL) western blotting detection reagents, Pierce, Rockford, IL, USA; diisopropylfluorophosphate (DFP), Fluka AG, Buchs, Switzerland; pertussis toxin, colchicine, lumicolchicine, taxol (paclitaxel), wortmannin, Sigma, Buchs, Switzerland. Gey’s medium contained 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM HEPES, pH 7.2, 0.1% HSA (wt/vol). In some experiments (see Results) $MgSO_4$ and $CaCl_2$ were omitted to prevent cell aggregation. HSA was omitted for determination of membrane association of proteins and for assays of protein phosphorylation. Y-27632 was kindly provided by Welfide Corporation (Osaka, Japan). Stock solutions of Y-27632 (10 mM) were prepared in H_2O and aliquots were stored at 4°C. Wortmannin and fNLPNTL were added as stock solutions in DMSO. DMSO alone, up to 0.4%, had no effect in our experiments.

Antibodies

A monoclonal antibody specific for RhoA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and a monoclonal antibody directed against Rokα was obtained from Transduction Laboratories (Lexington, KY). A polyclonal antibody specific for p42/44 MAPK phosphorylated on threonine and tyrosine was obtained from New England Biolabs, Beverly, MA. A polyclonal antibody specific for Akt phosphorylated on serine 473 and a polyclonal antibody reacting with Akt independent of its phosphorylation state were obtained from Cell Signaling Technology Inc. (Beverly, MA). A polyclonal antibody produced in rabbits against a peptide corresponding to myosin light chain (MLC) phosphorylated on Thr-16 and Ser-19 (PPMLC) (Ratcliffe et al., 1999) was kindly provided by J. M. Staddon (Eisai London Research Laboratories, London, UK). Goat anti-mouse and goat anti-rabbit antibodies conjugated to alkaline phosphatase were obtained from Bio-Rad (Richmond, CA).

Isolation of human neutrophils

Neutrophils were isolated from heparinized human blood (10 units/ml). In a first step, red blood cells were removed with a solution containing 12% (w/v) OptiPrep and 16.6 g/l methocel in 130 mM NaCl (Böyum, 1968). Subsequently, mononuclear cells were removed using NIM, which contains Hypaque and Ficoll (Ferrante and Thong, 1980). Approximately 95% of the leukocytes were neutrophils.

Analysis of shape changes

Neutrophils (3×10^6 cells/ml) were incubated in Gey’s medium containing 0.1% HSA but lacking divalent cations in a reciprocating waterbath at 37°C without or with inhibitors, colchicine, taxol or fNLPNTL, as indicated in Results. The reaction was stopped by fixing the cells in 1% glutaraldehyde (final concentration) for 30 minutes. The cells were washed and resuspended in 0.9% NaCl containing NaN_3 (1 mg/ml). Cell shape was determined using differential interference contrast microscopy (Nomarski optics) using a Zeiss IM 35 microscope with a 100× objective (NA 1.25). The shapes of neutrophils were classified into the following categories as previously described (Keller and Niggli, 1993): spherical (for examples, see Fig. 1, control), spherical with unifocal projections, polarized (for examples, see Fig. 1, fNLPNTL, 10 minutes) and non-polar cells with surface projections (for examples, see Fig. 1, fNLPNTL, 1 minute).

Treatment with pertussis toxin

Neutrophils (3×10^6/ml) were preincubated in Iscove’s medium with 10% fetal calf serum without or with pertussis toxin (400 ng/ml) for 2 hours at 37°C in a humidified atmosphere. After 2 hours, the cells were centrifuged and the cells resuspended in Gey’s medium with divalent cations, 0.5% HSA with or without pertussis toxin (450 ng/ml). Cell shape was then determined after incubation in the presence or absence of fNLPNTL or colchicine.

Chemokinesis

Neutrophils (4×10^6/ml, 0.45 ml per assay) were incubated in Gey’s medium containing divalent cations and 2% (experiments with colchicine) or 10% (experiments with fNLPNTL) HSA with inhibitors and/or stimuli at 37°C in a reciprocating waterbath as indicated in Results. The cells were centrifuged at 300 ***g*** for 5 minutes and resuspended in 50 μl of the supernatant. Neutrophils (5 μl containing 0.18×10^6 cells) were placed between a slide and a round coverslip (25 mm diameter). The slide-coverslip preparation was sealed with paraffin and placed on the heated stage (37°C) of a Zeiss IM 35 microscope with a 63× objective. The locomotor behavior of the cells (7-33 cells for each condition) was recorded for 10 minutes using video microscopy. The outline of the cell at the initial and final

position and the path traveled during the 10 minutes was drawn on a transparency. Cells remaining totally or partially within the outline of the initial position are defined as stationary; cells found outside of the outline after 10 minutes are defined as locomoting. The length of the path of individual cells was measured using a KS-300 analysis system (Kontron, Eching, Germany).

Neutrophil fractionation and detection of RhoA and Rokα

After treatment with DFP to block endogenous proteases (Amrein and Stossel, 1980), neutrophils (12×10^6 cells/ml) were resuspended in Gey's medium without divalent cations and HSA. Aliquots (450 μl) of this suspension were incubated with inhibitors and/or stimuli as described in Results. The reaction was stopped by centrifugation (300 ***g***, 5 minutes, 4°C). Cells were then resuspended in 500 μl relaxation buffer (Dusi et al., 1996) containing 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM EGTA, 10 mM Pipes, pH 7.3, 1 mM EDTA, 10 mM NaF. This suspension was incubated for 5 minutes on ice followed by sonication (Branson tip sonifier B-10, output 3.5, 1×10 seconds). After addition of 4 mM DFP to the cell lysate and a low-speed centrifugation for 5 minutes at 700 ***g*** and 4°C to remove unbroken cells and nuclei, the supernatants were centrifuged at 100,000 ***g*** for 30 minutes at 4°C in a Beckmann table top ultracentrifuge. The resulting pellets (corresponding to the membrane fraction) were dissolved in 100 μl of Laemmli sample buffer (Laemmli, 1970) by incubation at 95°C with vortexing for 10 minutes. The solubilized pellets, together with aliquots of the high-speed supernatants (soluble cytosolic fractions), were subjected to electrophoresis on 5-10% (for Rokα) or 5-20% (for RhoA) SDS-polyacrylamide gradient gels and transblotted to nitrocellulose using a mini-genie blotter from Idea Scientific (Minneapolis, MN) for 70 minutes, 24 Volts. Blots were exposed to antibodies to RhoA (diluted 1:1000) or to Rokα (diluted 1:500). For visualization of bound antibody an anti-mouse antibody conjugated to alkaline phosphatase, diluted 1:7000, followed by ECL detection of the second antibody, was used. The bands were scanned with a Camag TLC scanner at 590 nm and the area of the peaks was taken as a relative measure for the amount of protein present in the bands.

Analysis of phosphorylation of p42/44 MAPK, Akt or MLC in neutrophils

Neutrophils in 0.45 ml Gey's medium lacking divalent cations and HSA (0.5×10^6 cells for analysis of p42/44 MAPK, 1.5×10^6 cells for analysis of Akt and 2×10^6 cells for analysis of PPMLC) were exposed to stimuli as described in Results. The reaction was stopped by addition of 0.5 ml of a solution containing 20% (w/v) trichloroacetic acid (TCA), 40 mM NaF and 10 mM Na_2HPO_4. After a 20 minute incubation on ice, the precipitates were collected by centrifugation and washed once with 0.5 ml of 5% TCA and once with 0.5% TCA. The pellets were solubilized in sample buffer (see above), followed by separation of proteins on a 5-10% SDS-polyacrylamide gradient gel for analysis of p42/44 MAPK, a 10% gel for Akt and a 15% gel for analysis of PPMLC. Phosphorylated proteins were detected using a polyclonal antibody specific for p42/44 MAPK phosphorylated on threonine and tyrosine, diluted 1:1000, or a polyclonal antibody specific for Akt phosphorylated on serine 473, diluted 1:1000, or a polyclonal antibody specifically reacting with PPMLC, diluted 1:500. For visualization of PPMLC, blots were blocked in PBS containing 2% BSA and 0.05% Tween, followed by incubation with the primary antibody overnight at 4°C and a 1 hour incubation with the goat anti-rabbit IgG antibody prior to ELC detection. Detection of phosphorylated Akt was carried out as recommended by the producer.

Statistical analysis of data

Differences between data were analyzed with the Student's *t*-test for paired data with a P value of <0.05 considered significant. Data correspond to the mean±s.e.m.

Results

Stabilization of microtubules by taxol prevents colchicine-induced development of polarity and migration and also partially suppresses chemotactic-peptide-induced migration

First, we tested whether stabilization of microtubules prevents the effects of colchicine on cell shape and migration. As shown in Fig. 1, incubation of neutrophils with 10 μM colchicine for 30 minutes induces polarity in up to 80% of the cells. The morphology of colchicine-stimulated cells is comparable to that of cells stimulated with chemotactic peptide, although in colchicine-induced cells ruffling and formation of veils in the leading edge is reduced (Fig. 1) (Keller et al., 1984). Preincubation of cells with 1 μM taxol completely suppressed colchicine-induced development of polarity (Figs 1 and 2). The cells assumed a spherical shape with a smooth surface that is indistinguishable from that of the controls. Up to 20 μM taxol did not affect morphology of resting cells and also did not significantly affect morphology of cells stimulated with chemotactic peptide (Fig. 2). Inhibition of colchicine-induced polarity correlates with a 96±4% inhibition of colchicine-induced migration by 1 μM taxol (Table 1A). Although taxol did not affect polarity of chemotactic-peptide-activated cells (Fig. 2), this drug significantly reduced the fraction of cells migrating in the presence of fNLPNTL by 81±6% (n=4) (Table 1B). Cells preincubated with taxol prior to exposure to chemotactic peptide tended to be more elongated, with the rear of the cell adhering to the substrate (data not shown).

Colchicine-induced motile responses in neutrophils are suppressed by inhibition of Rho-kinase but are insensitive to pertussis toxin and the PI 3-kinase inhibitor wortmannin

On the basis of the data obtained in fibroblasts where microtubule disruption activates Rho (Ren et al., 1999), we investigated a possible role for the Rho-effector Rho-kinase in colchicine-induced development of polarity and migration in human neutrophils. Preincubation of cells with the Rho-kinase inhibitor Y-27632 suppressed development of polarity in a concentration-dependent manner (Figs 1 and 3), which is comparable to effects on chemotactic-peptide-stimulated cells (Niggli, 1999). Maximal inhibition was obtained at 10 μM of the inhibitor (95±3% inhibition, n=5). Half-maximal values were obtained at 2.6±0.8 μM (n=5), which is comparable to the concentration necessary for inhibition of the purified enzyme (Uehata et al., 1997). As shown in Fig. 1, most of the cells preincubated with 10 μM Y-27632 before addition of colchicine were spherical, although a few cells still showed small projections. This is different from chemotactic-peptide-stimulated cells pretreated with Y-27632, which still exhibit large F-actin-rich ruffles (Niggli, 1999). We also assessed the effects of Y-27632 on colchicine-induced chemokinesis and observed a highly significant (P<0.0025) reduction of 76±9% of the fraction of migrating cells (Table 2). The speed of migration of the remaining

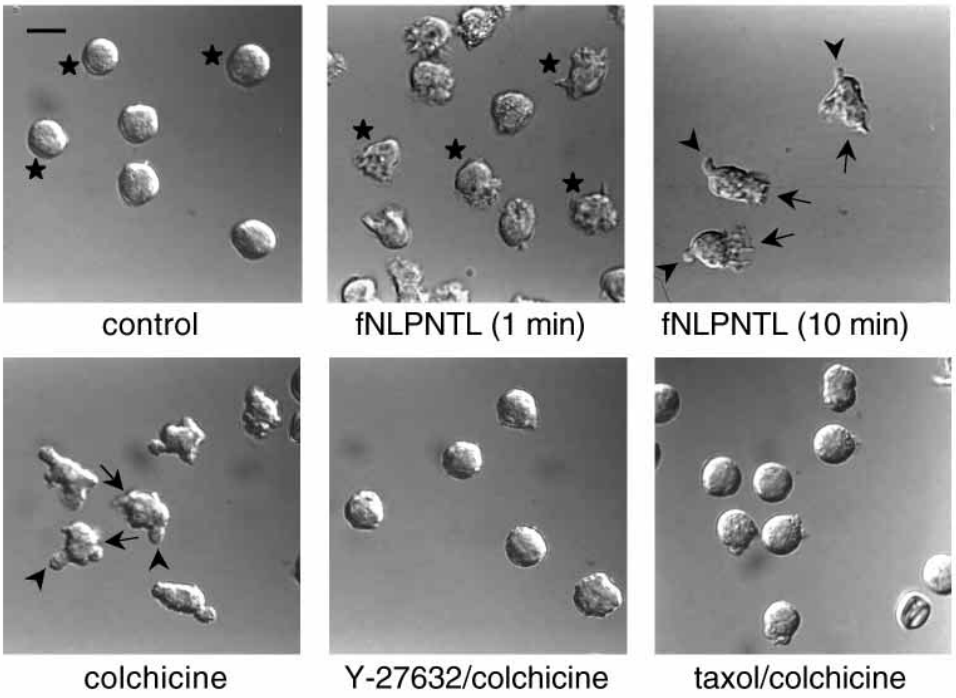

Fig. 1. Morphology of neutrophils exposed to chemotactic peptide or to colchicine without or with preincubation with taxol or the Rho-kinase inhibitor Y-27632. Neutrophils were preincubated for 30 minutes at 37°C followed by a further incubation either in medium for 30 minutes (control) or in medium containing 1 nM fNLPNTL for 1 or 10 minutes or in 10 μM colchicine for 30 minutes. Alternatively, cells were preincubated for 30 minutes in the presence of 10 μM Y-27632 or with 1 μM taxol followed by addition of colchicine (10 μM) and a further incubation for 30 minutes. Cells were fixed with glutaraldehyde and examined using Nomarski optics. Bar, 10 μm. Asterisks indicate typical examples of spherical cells in the control panel and of non-polar cells with ruffles in the panel showing cells stimulated for one minute with fNLPNTL. Arrows indicate the front with protrusions (pseudopods, ruffles), arrowheads the contracted tail (uropod) of typical examples of polarized cells. Note that the cells polarized by colchicine show less ruffling at the front than those stimulated with fNLPNTL

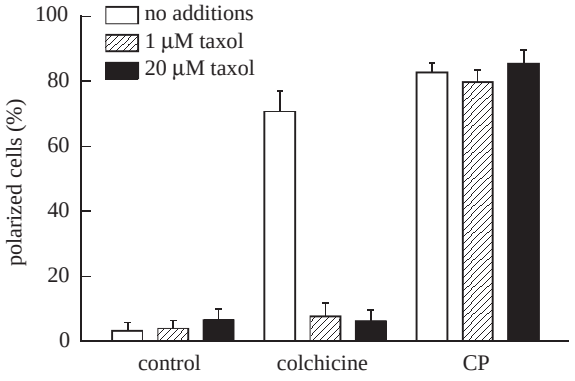

Fig. 2. Stabilization of microtubules with taxol prevents colchicine-induced development of polarity. For controls, neutrophils were either incubated for 60 minutes in medium (open bars) or preincubated for 30 minutes in medium followed by addition of 1 μM (hatched bars) or 20 μM taxol (closed bars) and a further incubation at 37°C. For cells stimulated with colchicine, cells were incubated with DMSO (open bars) or with 1 μM (hatched bars) or 20 μM taxol (closed bars) for 30 minutes at 37°C, followed by the addition of colchicine (10 μM) and a further incubation for 30 minutes. For cells stimulated with the chemotactic peptide fNLPNTL (CP), cells were preincubated for 30 minutes with DMSO (open bars) or with 1 μM (hatched bars) or 20 μM taxol (closed bars) at 37°C followed by addition of 1 nM fNLPNTL and a further incubation for 30 minutes. Cells were fixed with glutaraldehyde and the percentage of polarized cells assessed using Nomarski optics for 100 cells per sample. Mean±s.e.m. of four experiments.

motile cells was also significantly (P<0.005) decreased by 66-86%. These findings suggest that microtubule disassembly activates the Rho/Rho-kinase cascade in neutrophils and that this process is required for colchicine-induced neutrophil migration.

We assessed the effect of inhibiting activity of heterotrimeric Gi proteins with pertussis toxin on chemotactic-peptide- and colchicine-induced development of polarity. As shown in Fig. 4, preincubation with 400 ng pertussis toxin/ml completely abolished chemotactic peptide-induced polarity. The cells assumed the spherical morphology typical of resting cells. By contrast, colchicine-induced polarity was not affected, suggesting that microtubule disassembly does not activate motile processes in neutrophils via Gi.

PI 3-kinase plays an important role in chemotactic-factor-induced neutrophil migration (Hirsch et al., 2000; Stephens et al., 2002). As shown in Fig. 5A and in our previous work (Niggli and Keller, 1997), the PI 3-kinase inhibitor wortmannin markedly reduces chemotactic-peptide-induced development of polarity and migration in human neutrophils, correlating with inhibition of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] production. Half-maximal effects of wortmannin on cell polarization were obtained in the range of 14-32 nM of the drug. Development of polarity was inhibited by 58±8% (n=5) by 100 nM wortmannin (Fig. 5A) (Niggli and Keller, 1997). We assessed whether the incomplete inhibition of chemotactic-peptide-induced cell

Table 1. Effect of taxol on (A) colchicine- and (B) chemotactic-peptide-induced cell migration

Additions	Migrating cells (%)	Mean speed of migrating cells (μm/minute)
A*		
Medium	0±0	–
Colchicine	80±4	8±2
Taxol/colchicine	4±4	1
B†		
Medium	4±3	2±1
fNLPNTL	54±14	7±1
Taxol/fNLPNTL	9±3	5±2

*Neutrophils were preincubated for 30 minutes without or with 1 μM taxol at 37°C, followed by addition of 10 μM colchicine and a further incubation for 30 minutes. †Neutrophils were preincubated for 30 minutes without or with 10 μM taxol at 37°C, followed by addition of 1 nM fNLPNTL and a further incubation for 30 minutes.

Subsequently locomotor activity was determined in slide-coverslip preparations at 37°C using video microscopy (A: 7-15 cells per sample; B: 8-26 cells per sample). Mean±s.e.m. of three to four independent experiments.

polarization by wortmannin is due to incomplete inhibition of PI 3-kinase by monitoring the effect of wortmannin on activation of a major target of PI 3-kinase, the serine-threonine protein kinase Akt (or protein kinase B; PKB). Hirsch et al. showed that chemotactic-peptide-induced increased phosphorylation of Akt is completely abolished in neutrophils of mice lacking PI 3-kinase γ, whereas chemotaxis of neutrophils from these mice is only partially impaired (Hirsch et al., 2000). We now show that wortmannin (100 nM) also completely suppressed fNLPNTL-induced Akt phosphorylation in human neutrophils (Fig. 5B). By contrast, this concentration of wortmannin did not affect colchicine-induced development of polarity (Fig. 5A). Moreover we could not detect any Akt phosphorylation in cells treated with colchicine for 5 to 30 minutes, whereas exposure of cells to 1 nM fNLPNTL for 5 minutes induced a marked signal (Fig. 5B).

Previous data suggested that microtubule disruption activates p42/44 MAPK rapidly in human pro-monocytic cells (Schmid-Alliana et al., 1998). However, we could not observe a significant increase in phosphorylation of p42/44 MAPK in neutrophils exposed to colchicine for 5-30 minutes, whereas activation with fNLPNTL for 5 minutes had a very marked effect (Fig. 6).

Table 2. Effect of Rho-kinase inhibition by Y-27632 on colchicine-induced cell migration

Additions	Migrating cells (%)	Mean speed of migrating cells (μm/minute)
Medium	4±2	2.1±0.4
Colchicine	79±6	8.3±0.3
Y-27632/colchicine	20±8	2.6±1.4

Neutrophils were preincubated for 30 minutes without or with 10 μM Y-27632 at 37°C, followed by addition of 10 μM colchicine and a further incubation for 30 minutes. Subsequently, locomotor activity was determined in slide coverslip preparations at 37°C using video microscopy (15-33 cells per sample). Means±s.e.m. of three independent experiments.

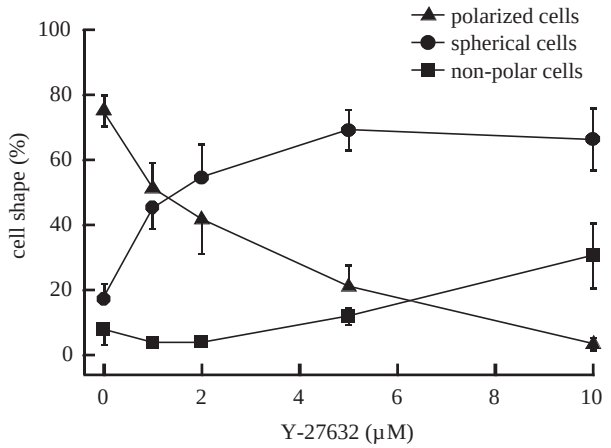

Fig. 3. Concentration-dependent effect of the Rho-kinase inhibitor Y-27632 on the morphology of colchicine-stimulated neutrophils. Neutrophils were preincubated for 30 minutes without or with increasing amounts of Y-27632, as indicated, at 37°C. The incubation was continued for another 30 minutes either in medium or in 10 μM colchicine. Cells were fixed with glutaraldehyde and examined using Nomarski optics. The percentage of spherical cells (circles), cells with front-tail polarity (triangles) and non-polar cells (squares) was determined for 100 cells per sample. In the absence of colchicine and Y-27632, 96±4% of the cells were spherical, 3±3% polarized and 2±1% non-polar. Mean±s.e.m. of five experiments.

Rho and Rho-kinase are translocated to the membrane in neutrophils exposed to colchicine or chemotactic peptide

Translocation of Rho-kinase to the plasma membrane may correlate with its activation (Taggart et al., 1999). Therefore we studied the effect of colchicine on membrane association of Rokα (Rho-kinase II) in human neutrophils and compared it with the effects of chemotactic peptide. In resting cells,

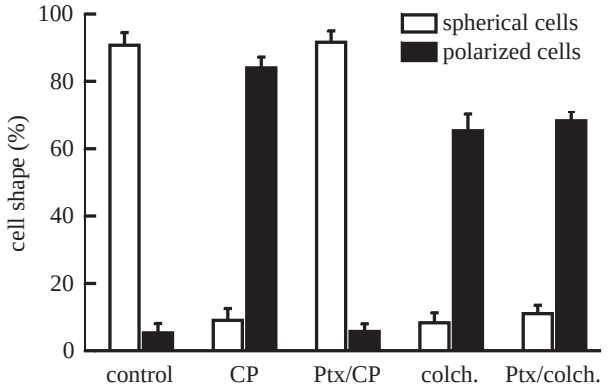

Fig. 4. Pertussis toxin selectively inhibits chemotactic-peptide- but not colchicine-induced development of polarity. Neutrophils were treated without or with 450 ng/ml pertussis toxin (Ptx) as described in Materials and Methods, followed by resuspension in Gey's medium and incubation without (control) or with 1 nM fNLPNTL (CP) or with 10 μM colchicine (colch.) for 30 minutes at 37°C. Cells were fixed with glutaraldehyde and the percentage of spherical (open bars) and polarized cells (closed bars) assessed using Nomarski optics for 100 cells per sample. Mean±s.e.m. of three experiments.

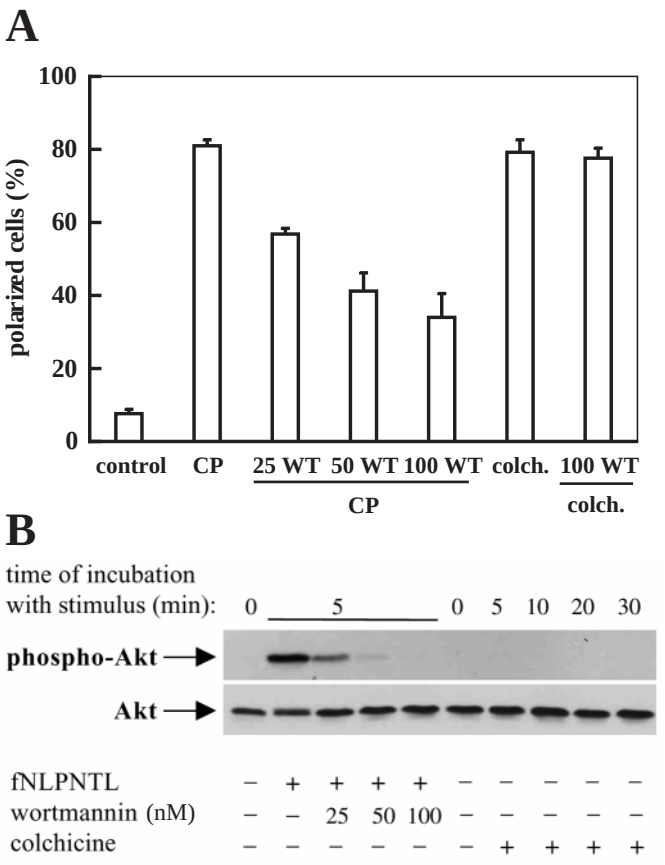

Fig. 5. PI 3-kinase is involved in chemotactic-peptide- but not in colchicine-induced development of polarity. (A) Effect of wortmannin on chemotactic-peptide- and colchicine-induced polarity. Neutrophils were preincubated for 10 minutes with DMSO or with 25, 50 or 100 nM wortmannin (WT) at 37°C. The incubation was continued for another 30 minutes in medium (control) or with 10 μM colchicine (colch.) or with 1 nM fNLPNTL (CP). Cells were fixed with glutaraldehyde and the percentage of polarized cells assessed using Nomarski optics for 100 cells per sample. Mean±s.e.m. of five experiments. (B) Akt phosphorylation in response to chemotactic peptide and colchicine. Stimulation with chemotactic peptide: neutrophils were preincubated for 10 minutes with DMSO or with 25, 50 or 100 nM wortmannin at 37°C. The incubation was continued for another 5 minutes in the absence or presence of 1 nM fNLPNTL. Stimulation with colchicine: neutrophils were either incubated for 40 minutes at 37°C in medium or were exposed, after preincubation in medium for 10-35 minutes, for the times indicated to 10 μM colchicine (total incubation time: 40 minutes). Cells were precipitated with TCA and subjected to immunoblotting in order to visualize phosphorylated Akt (phospho-Akt). The blot was then stripped and reprobed with an antibody that reacts with Akt independently of its activation state. Immunoblots representative of three experiments are shown.

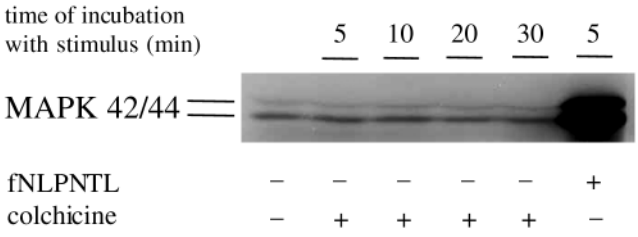

Fig. 6. Treatment with colchicine does not induce phosphorylation of p42/44 MAPK in neutrophils. Neutrophils were either incubated for 40 minutes at 37°C in medium or were exposed, after preincubation in medium for 10-35 minutes, for the times indicated to 1 nM fNLPNTL or to 10 μM colchicine (total incubation time: 40 minutes). Cells were precipitated with TCA and subjected to immunoblotting in order to visualize phosphorylated p42/44 MAPK. An immunoblot representative of four experiments is shown. Note, for the control and the samples incubated with colchicine, 1.8×10^5 cells were applied per lane; for the sample incubated with fNLPNTL, 0.2×10^6 cells were applied per lane.

12±7% (n=3) of total enzyme was recovered in the membrane-containing fraction. Incubation of cells with colchicine (5 minutes) induced a marked, 99±20% increase (n=7, P<0.0025) in membrane-associated Rokα, which is comparable with that induced by chemotactic peptide (171±50% increase, n=3), as shown in Fig. 7A,B. Membrane association of Rokα was maximal 5 minutes after addition of colchicine and decreased to a lower level, which was still significantly higher than control levels 10 minutes after addition of the drug (data not shown). This response is thus more transient than the effect of colchicine on cell polarity where 93% of the maximal polarization response was observed 5 minutes after addition of colchicine and 100% 10 minutes after addition of the drug. Similar to effects on cell shape and migration (Fig. 2, Table 1), preincubation with taxol also completely prevented colchicine-induced membrane association of Rokα (Fig. 7A,B), whereas that induced by chemotactic peptide was only partially affected (63±16% inhibition, n=6, Fig. 7C,D). In the latter case the extent of inhibition was quite variable in different experiments. Lumicolchicine, which is inactive on microtubules, did not induce membrane association of Rokα (Fig. 7A,B).

Activation of neutrophils with chemotactic peptide induces recruitment of a fraction of Rho and Rac from the cytosol to the membrane (Dusi et al., 1996; Marcil et al., 1999). Incubation of cells with 10 μM colchicine for 5 minutes also induced a significant shift of RhoA to the membrane (50-230% increase). This increase was comparable to that induced by a 30 minutes incubation with 1 nM fNLPNTL (102±47% increase) (data not shown).

Our data strongly suggest that microtubule disassembly in neutrophils activates RhoA and its effector Rokα.

Microtubule disassembly induces phosphorylation of myosin light chain

We wanted to obtain information on downstream targets of Rho-kinase activated in cells by colchicine. As shown previously, exposure to chemotactic peptide induces a sustained increase in myosin light chain phosphorylation in human neutrophils that can be abolished by the Rho-kinase inhibitor Y-27632 (Niggli, 1999). As assessed with an antibody that specifically reacts with myosin light chain phosphorylated on Thr-16 and Ser-19 (PPMLC) (Ratcliffe et al., 1999), treatment of neutrophils with 10 μM colchicine induced a time-dependent increase in the amount of PPMLC; this increase was

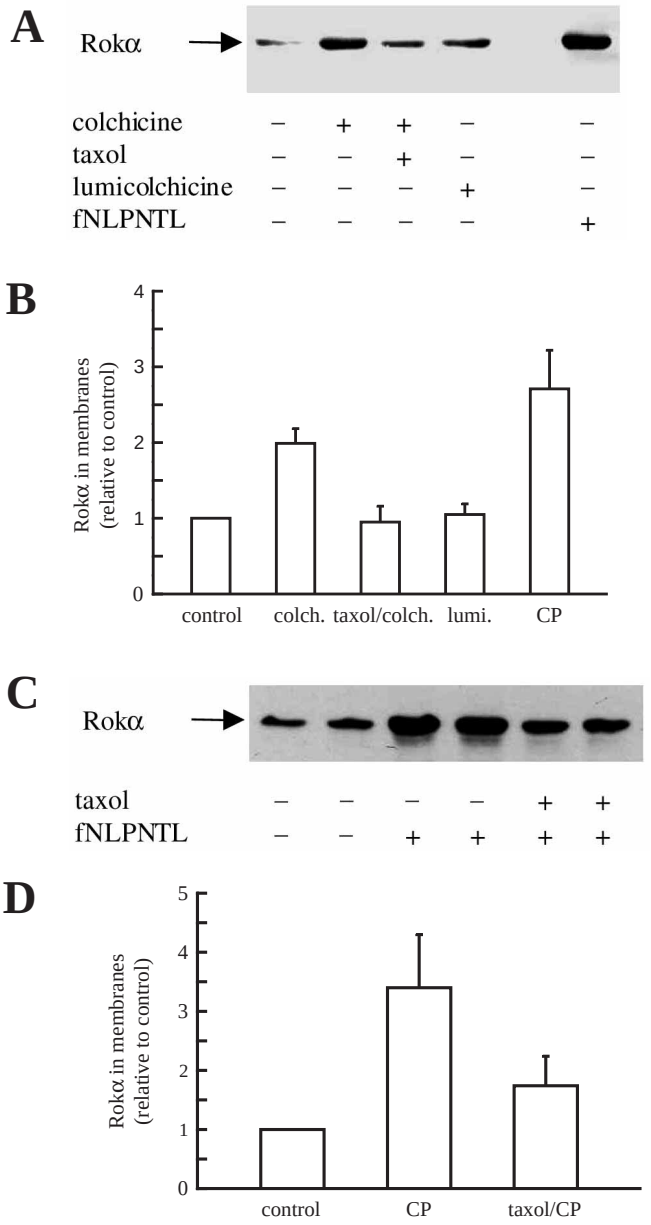

Fig. 7. Translocation of Rokα from cytosol to the membrane in neutrophils treated with colchicine or chemotactic peptide. (A,B) Neutrophils were preincubated for 15 minutes at 37°C without or with 10 μM taxol. The incubation was continued for another 5 minutes in medium (control) or with 10 μM colchicine (colch.) or with 10 μM lumicolchicine (lumicolch.). For the incubation with chemotactic peptides, cells were preincubated for 5 minutes at 37°C, followed by addition of 1 nM fNLPNTL (CP) and a further incubation for 15 minutes. Membrane and cytosolic fractions were subsequently prepared and analyzed by immunoblotting for the presence of Rokα (arrow). (A) Immunoblot of a typical experiment; (B) Quantitative evaluation of A (mean±s.e.m. of three experiments; cells with colchicine: n=7). (C,D) Neutrophils were preincubated for 15 minutes at 37°C, without or with 10 μM taxol. The incubation was continued for another 15 minutes either in medium (control) or with 1 nM fNLPNTL (CP) and a further incubation for 15 minutes. The amount of Rokα present in the membrane fraction was then analyzed by immunoblotting. (C) Immunoblot of a typical experiment (duplicates of each assay are shown); (D) quantitative evaluation of C (mean±s.e.m. of six experiments).

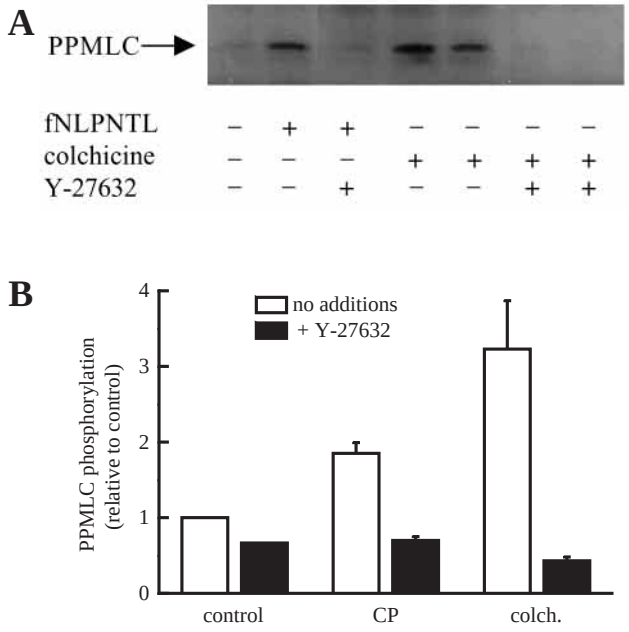

Fig. 8. Colchicine treatment increases myosin light chain phosphorylation in neutrophils in a Y-27632-sensitive manner. Neutrophils were either incubated for 50 minutes at 37°C in medium or in 10 μM Y-27632 or were preincubated in the absence or presence of 10 μM Y-27632 for 30 minutes followed by a further incubation with 1 nM fNLPNTL for 5 minutes or with 10 μM colchicine for 20 minutes as indicated. Cells were precipitated with TCA and subjected to immunoblotting. (A) Immunoblot of a representative experiment (for cells exposed to colchicine, duplicates are shown). (B) Quantitative evaluation of A (mean±s.e.m. of five to six experiments; control with Y-27632: n=2).

maximal 20-30 minutes after addition of the drug (a 223±65% increase, n=5 at 20 minutes, P<0.025; Fig. 8). 34 to 57% of the response was attained 5 minutes after addition of the drug to the cells. As shown in Fig. 8, the maximal increase obtained 20 minutes after addition of colchicine was even higher than that induced by a 5 minute incubation with chemotactic peptide and could be completely abolished by preincubation of the cells with 10 μM Y-27632. Preincubation with 10 μM taxol also completely abolished the colchicine-induced increase in PPMLC (data not shown).

Discussion

Disruption of the neutrophil microtubule network induces development of polarity and chemokinesis in human neutrophils comparable to the effects of chemotactic peptide. We confirm here that these effects are due to microtubule disruption and not to non-specific effects of colchicine as they can be prevented by the addition of taxol, which stabilizes microtubules, and lumicolchicine, which is inactive on microtubules, did not affect neutrophil shape and migration (Keller et al., 1984). We provide now data on the signalling pathways involved in microtubule-induced development of polarity and migration in neutrophils and on a downstream target.

Differential signalling pathways are involved in microtubule-disruption- and in chemotactic-peptide-induced neutrophil polarity and migration

We demonstrate here that neutrophil motility induced by microtubule disruption is probably not due to direct effects on actin organization; instead it is probably due to activation of specific signalling pathways. We studied four different signalling pathways that could conceivably be affected by microtubule disruption: heterotrimeric G-proteins, the Rho-Rho-kinase pathway, the PI 3-kinase pathway and activation of p42/44 MAPK. Concerning heterotrimeric G-proteins, membrane-bound tubulin has been reported to directly activate Gαs, Gαi1 and Gαq (Popova et al., 1997). However, Gi inhibition in neutrophils by pertussis toxin did not suppress colchicine-induced cell polarization in our system, which excludes G_i proteins from having a role in this response. Chemotactic-peptide-induced shape changes in contrast were completely prevented by pertussis toxin.

We have shown previously that inhibition of Rho-kinase activity results in almost complete suppression of chemotactic-peptide-induced polarization and migration of human neutrophils (Niggli, 1999). We now show that Rho-kinase inhibition also almost completely suppresses colchicine-induced development of polarity and markedly inhibits colchicine-induced chemokinesis in neutrophils. This finding correlates with reports showing that microtubule disassembly activates the Rho-Rho-kinase pathway in other cell types (Witman and Waterman-Storer, 2001). Indeed we could show that colchicine treatment results in increased membrane association of Rho-kinase, which can be prevented by taxol-based stabilization of microtubules (Fig. 6). Previous evidence obtained in smooth muscle cells suggests that membrane association of Rho-kinase correlates with its activation. There, carbachol stimulates association of Rho-kinase with the plasma membrane as demonstrated using immunofluorescence staining (Taggart et al., 1999). We now show that this also occurs in stimulated neutrophils exposed to chemotactic peptide or colchicine. We also provide evidence that Rho-kinase, activated by microtubule disassembly, induces a marked sustained increase in myosin light chain phosphorylation, comparable to that induced by exposure of cells to chemotactic peptide, which then leads to tail contraction and is crucial for colchicine-induced migration. Interestingly, the time course of colchicine-induced membrane association of Rho-kinase is somewhat faster than that of myosin light chain phosphorylation and development of polarity. Moreover the first event is transient, whereas the two other events are sustained for up to at least 30 minutes after addition of the stimulus. Transient activation of Rho-kinase thus results in sustained myosin light chain phosphorylation and polarization. Rho-kinase is thought to act by phosphorylating the myosin-binding subunit of myosin light chain phosphatase, thereby inhibiting this enzyme (Somlyo and Somlyo, 2000). A transient activation of Rho-kinase may be sufficient to inactivate the phosphatase for longer. This suggests that phosphatases acting on the myosin light chain phosphatase are inactive in the migrating cells. Interestingly, the phosphorylated myosin-binding subunit of type 1 protein phosphatase is highly resistant to dephosphorylation (Takizawa et al., 2002). Alternatively, activation of Rho-kinase may be maintained even after its dissociation from the membrane.

Regulatory subunits of PI 3-kinase interact with tubulin (Inukai et al., 2000). Activity of this enzyme could thus be affected by microtubule disassembly. However, colchicine-induced polarization was not sensitive to treatment with concentrations of the PI 3-kinase inhibitor wortmannin, which completely suppress chemotactic-peptide-induced phosphorylation of a major target of PI 3-kinase, Akt (Fig. 5B), and which markedly inhibit neutrophil migration induced by exposure to chemotactic peptide or to membrane-permeable PtdIns(3,4,5)P_3 (Niggli and Keller, 1997; Niggli, 2000). This strongly suggests that colchicine does not act via PI 3-kinase. In line with this notion, stimulation of cells with colchicine did not induce any detectable Akt phosphorylation (Fig. 5B).

A substantial fraction of p42/44 MAPK is associated with microtubules, and microtubule disruption activates these enzymes within minutes of addition of colchicine in human pro-monocytic cells (Schmid-Alliana et al., 1998). However, this was not the case in human neutrophils, suggesting differential regulation of different signalling systems depending on the cell type.

Our data strongly suggest that microtubule disassembly induces a selective activation of the Rho-Rho-kinase pathway but not of PI 3-kinase or of p42/44 MAPK. This activation of Rho-kinase, resulting in increased phosphorylation of myosin light chain on Thr-18 and Ser-19, is required for the colchicine-induced development of polarity and the subsequent migratory response in neutrophils. Concerning the speed of migration and the fraction of migrating cells, this response compares well with that induced by chemotactic peptides. However, in colchicine-induced cells, less ruffling occurs at the leading edge (Fig. 1) (Keller et al., 1984). This may be due to the lack of activation of PI 3-kinase and lack of local accumulation of PtdIns(3,4,5)P_3, which is thought to be crucial for chemotactic-peptide-induced polarity in neutrophils (Knall et al., 1997; Niggli and Keller, 1997; Niggli, 2000; Hirsch et al., 2000; Weiner, 2002; Stephens et al., 2002). Migration is thus not obligatorily coupled to localized production of PtdIns(3,4,5)P_3.

Concerning the mechanism of microtubule-disassembly-induced Rho activation, evidence has recently been provided for a direct regulation of the nucleotide exchange factor GEF-H1 by interaction with microtubules. This factor appears to be inactive when bound to microtubules. It is activated by microtubule disassembly and selectively activates Rho but not Rac1 or Cdc42 (Krendel et al., 2002).

Our data thus show that different stimuli activate migration in human neutrophils involving differential sets of signalling proteins depending on the initial stimulus.

Mechanisms of microtubule-disruption-induced cytoskeletal reorganization

Microtubule disassembly in neutrophils induces development of a polarized shape. The front of these polarized cells, as assessed by videomicroscopy, is defined as that part of the cell where protrusions are extended in the direction of migration, whereas pseudopod protrusion is suppressed in the rear of the cell, which is characterized by a narrow, contracted tail (Fig. 1) (Keller et al., 1984). Interestingly, polarization of cells induced by microtubule disassembly is accompanied by accumulation of F-actin and α-actinin in the front and myosin II in the tail; these results are similar to findings in chemotactic-

peptide-stimulated cells. Also, a small increase in the fraction of stable actin resistant to Triton X-100 could be detected in colchicine-treated cells (Keller and Niggli, 1993), which is similar to previous findings by another group (Tsai et al., 1998). Relatively small changes in the F-actin:G-actin ratio are thus sufficient to sustain rapid migration. The mechanism of this cytoskeletal reorganization remains to be clarified. We observed previously that Rho-kinase inhibition does not affect actin reorganization in human neutrophils (Niggli, 1999). Moreover, Rho inhibition in neutrophils did not suppress chemotactic-peptide-induced actin polymerization (Ehrengruber et al., 1995). However, we observed that in neutrophils pretreated with Y-27632 prior to addition of colchicine, F-actin was diffusely distributed in the cytosol, which is similar to results for untreated controls, suggesting a role for Rho-kinase in regulating the polarized accumulation of F-actin in colchicine-treated cells (data not shown).

Mechanisms of microtubule-disruption-induced development of polarity in neutrophils

Localized activation of signalling proteins and generation of diffusable inhibitors may be prerequisites for development of polarity and chemotaxis (Weiner, 2002; Stephens et al., 2002). Neutrophils develop polarity not only in gradients of chemoattractants but also in uniform concentrations of stimuli. In the latter case, stochastic differences in concentration of stimuli are thought to induce polarity. It is not clear how global depolymerization of microtubules induced by colchicine can result in localized activation of Rho/Rho-kinase and localized myosin activation presumably required for development of polarity. One would have to assume a fortuitous local higher activation of the Rho/Rho-kinase system, which would result in generation of a diffusable inhibitor and localized positive feedback. However such factors regulating the Rho/Rho-kinase system have not yet been identified in neutrophils (Stephens et al., 2002).

The role of microtubule disassembly in migration of chemotactic-peptide-stimulated cells

As outlined by Wittmann and Waterman-Storer, cells such as fibroblasts or macrophages require an intact microtubule network for development of polarity and migration whereas other cells such as neutrophils do not (Wittmann and Waterman-Storer, 2001). The exact role of microtubules in maintaining polarity in specific cell types is still under debate. Microtubules may be required for transport of membranes to the leading edge and/or for selectively stabilizing the leading edge and/or for regulating focal adhesion dynamics ensuring disassembly of focal contacts in the rear of the cell (Wittmann and Waterman-Storer, 2001). Neutrophils polarize not only when adhering to a substrate but also in suspension. Possibly the need for intact microtubules for fibroblast polarization is related to the role of cell substrate contacts in this process. Adhesion-independent polarization of neutrophils does not require intact microtubules. As microtubule disassembly on its own can induce a migratory response in neutrophils, the question arises whether localized microtubule disassembly induced by chemotactic peptide also plays a role in receptor-linked chemotactic responses. The microtubule-stabilizing drug taxol does not affect chemotactic-peptide-induced cell polarization in suspension but partially inhibits chemotactic-peptide-induced membrane association of Rho-kinase and markedly inhibits chemotactic-peptide-induced chemokinesis (Fig. 7C,D; Table 1B). The third observation is in agreement with earlier data showing abolishment of fMLP-induced chemotaxis in neutrophils by taxol (Roberts et al., 1982). According to these authors, taxol reduces spreading of neutrophils. Localized microtubulin depolymerization occurring during cell migration, which in turn results in localized Rho/Rho-kinase activation, may contribute to regulation of cell adhesion. The partial inhibition of Rho-kinase activation by taxol observed in chemotactic-peptide-stimulated cells may not be sufficient to abolish development of polarity in suspension but may significantly impair cell migration.

In summary, we provide novel data showing that microtubule disassembly in human neutrophils selectively activates the Rho/Rho-kinase pathway but not PI 3-kinase or p42/44 MAPK. Motility ensuing from microtubule disruption is independent of PI 3-kinase but requires Rho-kinase activity. This is in contrast to development of polarity and migration induced by chemotactic peptides or membrane-permeable PtdIns(3,4,5)P_3 where the PI 3-kinase pathway plays a major role. Differential sets of signalling pathways are thus required for migration depending on the initial stimulus.

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References

Amrein, P. C. and Stossel, T. P. (1980). Prevention of degradation of human polymorphonuclear leukocyte proteins by diisopropyl-fluorophosphate. *Blood* **56**, 442-447.

Böyum, A. (1968). Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**, 1-89.

Chung, C. Y., Funamoto, S. and Firtel, R. A. (2001). Signaling pathways controlling cell polarity and chemotaxis. *Trends Biochem. Sci.* **26**, 557-566.

Dusi, S., Donini, M. and Rossi, F. (1996). Mechanisms of NADPH oxidase activation: translocation of p40phox, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47phox or p67phox. *Biochem. J.* **314**, 409-412.

Dziezanowski, M. A., DeStefano, M. J. and Rabinovitch, M. (1980). Effect of antitubulins of spontaneous and chemotactic migration of neutrophils under agarose. *J. Cell Sci.* **42**, 379-388.

Ehrengruber, M. U., Boquet, P., Coates, T. D. and Deranleau, D. A. (1995). ADP-ribosylation of Rho enhances actin polymerization-coupled shape oscillations in human neutrophils. *FEBS Lett.* **372**, 161-164.

Enomoto, T. (1996). Microtubule disruption induces the formation of actin stress fibers and focal adhesions in cultured cells: possible involvement of the Rho signal cascade. *Cell Struct. Funct.* **21**, 317-326.

Ferrante, A. and Thong, Y. H. (1980). Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque-Ficoll method. *J. Immunol. Methods* **36**, 109-117.

Hirsch, E., Katanaev, V. L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F. and Wymann, M. P. (2000). Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* **287**, 1049-1053.

Ingber, D. E. (1993). Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J. Cell Sci.* **106**, 613-627.

Inukai, K., Funaki, M., Nawano, M., Katagiri, H., Ogihara, T., Anai, M., Onishi, Y., Sakoda, H., Ono, H., Fukushima, Y. et al. (2000). The N-

terminal 34 residues of the 55 kDa regulatory subunits of phosphoinositide 3-kinase interact with tubulin. *Biochem. J.* **346**, 483-489.

Keller, H. U. and Niggli, V. (1993). Colchicine-induced stimulation of PMN motility related to cytoskeletal changes in actin, α-actinin, and myosin. *Cell Motil. Cytoskel.* **25**, 10-18.

Keller, H. U., Naef, A. and Zimmermann, A. (1984). Effects of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leukocytes. *Exp. Cell Res.* **153**, 173-185.

Knall, C., Worthen, G. S. and Johnson, G. L. (1997). Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proc. Natl. Acad. Sci. USA* **94**, 3052-3057.

Kolodney, M. S. and Elson, E. L. (1995). Contraction due to microtubule disruption is associated with increased phosphorylation of myosin regulatory light chain. *Proc. Natl. Acad. Sci. USA* **92**, 10252-10256.

Krendel, M., Zenke, F. T. and Bokoch, G. M. (2002). Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. *Nat. Cell Biol.* **4**, 294-301.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Liu, B. P., Chrzanowska-Wodnicka, M. and Burridge, K. (1998). Microtubule depolymerization induces stress fibers, focal adhesions, and DNA synthesis via the GTP-binding protein Rho. *Cell Adhes. Commun.* **5**, 249-255.

Marcil, J., Harbour, D., Houle, M. G., Naccache, P. H. and Bourgoin, S. (1999). Monosodium urate-crystal-stimulated phospholipase D in human neutrophils. *Biochem. J.* **337**, 185-192.

Niggli, V. (1999). Rho-kinase in human neutrophils: a role in signalling for myosin light chain phosphorylation and cell migration. *FEBS Lett.* **445**, 69-72.

Niggli, V. (2000). A membrane-permeant ester of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is an activator of human neutrophil migration. *FEBS Lett.* **473**, 217-221.

Niggli, V. and Keller, H. U. (1997). The phosphatidylinositol 3-kinase inhibitor wortmannin markedly reduces chemotactic peptide-induced locomotion and increases in cytoskeletal actin in human neutrophils. *Eur. J. Pharmacol.* **335**, 43-52.

Popova, J. S., Garrison, J. C., Rhee, S. G. and Rasenick, M. M. (1997). Tubulin, Gq, and phosphatidylinositol 4,5-bisphosphate interact to regulate phospholipase Cβ1 signaling. *J. Biol. Chem.* **272**, 6760-6765.

Ratcliffe, M. J., Smales, C. and Staddon, J. M. (1999). Dephosphorylation of the catenins p120 and p100 in endothelial cells in response to inflammatory stimuli. *Biochem. J.* **338**, 471-478.

Ren, X. D., Kiosses, W. B. and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **18**, 578-585.

Rickert, P., Weiner, O. D., Wang, F., Bourne, H. R. and Servant, G. (2000). Leukocytes navigate by compass: roles of PI3Kγ and its lipid products. *Trends Cell Biol.* **10**, 466-473.

Roberts, R. L., Nath, J., Friedman, M. M. and Gallin, J. I. (1982). Effects of taxol on human neutrophils. *J. Immunol.* **129**, 2134-2141.

Schmid-Alliana, A., Menou, L., Manié, S., Schmid-Antomarchi, H., Millet, M., Giuriato, S., Ferrua, B. and Rossi, B. (1998). Microtubule integrity regulates Src-like and extracellular signal-regulated kinase activities in human pro-monocytic cells. *J. Biol. Chem.* **273**, 3394-3400.

Somlyo, A. P. and Somlyo, A. V. (2000). Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol.* **522**, 177-185.

Stephens, L., Ellson, C. and Hawkins, P. (2002). Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Curr. Opin. Cell Biol.* **14**, 203-213.

Taggart, M. J., Lee, Y. H. and Morgan, K. G. (1999). Cellular redistribution of PKCα, rhoA and Rokα following smooth muscle agonist stimulation. *Exp. Cell Research* **251**, 92-101.

Takizawa, N., Niiro, N. and Ikebe, M. (2002). Dephosphorylation of the two regulatory components of myosin phosphatase, MBS and CPI17. *FEBS Lett.* **515**, 127-132.

Tanaka, E., Ho, T., Kirschner, M. W. (1995). The role of microtubule dynamics in growth cone motility and axonal growth. *J. Cell Biol.* **128**, 139-155.

Tsai, M. A., Waugh, R. E. and Keng, P. C. (1998). Passive mechanical behavior of human neutrophils: effects of colchicine and paclitaxel. *Biophys. J.* **74**, 3282-3291.

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. and Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**, 990-994.

Vasiliev, J. M., Gelfand, I. M., Domnina, L. V., Ivanova, O. Y., Komm, S. G. and Olshevskaja, L. V. (1970). Effect of colcemid on the locomotory behaviour of fibroblasts. *J. Embryol. Exp. Morphol.* **24**, 625-640.

Waterman-Storer, C. M, Worthylake, R. A., Liu, B. P., Burridge, K. and Salmon, E. D. (1999). Microtubule growth activates Rac1 to promote lamellipodial protrusion in fibroblasts. *Nat. Cell Biol.* **1**, 45-50.

Weiner, O. D. (2002). Regulation of cell polarity during eukaryotic chemotaxis: the chemotactic compass. *Curr. Opin. Cell Biol.* **14**, 196-202.

Wittmann, T. and Waterman-Storer, C. M. (2001). Cell motility, can Rho GTPases and microtubules point the way? *J. Cell Sci.* **114**, 3795-3803.

Zigmond, S. H. (1977). Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J. Cell Biol.* **75**, 606-616.