ADP-ribosylation of Rho enhances actin polymerization-coupled shape oscillations in human neutrophils

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Abstract Stimulated neutrophils exhibit coordinated sinusoidal oscillations in filamentous actin content and cellular shape. We investigated the effect of inhibition of the small G protein Rho on neutrophil actin polymerization, shape changes and oscillations using a genetically engineered toxin that enters cells and selectively ADP-ribosylates endogenous Rho. This treatment increased the amplitudes and frequencies of shape oscillations and duration of the oscillating transient. However, it had no effect on the initial actin polymerization and shape changes induced by *N*-formyl-Met-Leu-Phe. Regulation of these oscillations may be important for the control of neutrophil motility.

Key words: Cytoskeleton; Polymorphonuclear leukocyte; Turbidimetry; G protein; Exoenzyme C3; Wortmannin

1. Introduction

Actin reorganization plays a central role in mediating neutrophil shape changes [1,2]. The signal transduction pathway for actin polymerization depends on agonist-receptor binding [1,2] and activation of a plasma membrane-located heterotrimeric G protein [3]. However, the events downstream of receptor-G protein interaction are a matter of debate. It was shown in human neutrophils that the transient increase in filamentous actin (F-actin) is parallelled by a rise in intracellular phosphatidylinositol 3,4,5-trisphosphate [4]. The authors concluded that stimulation activates phosphatidylinositol 3-kinase (PI 3-kinase), thereby generating phosphatidylinositol 3,4,5-trisphosphate which they suggested is an initiator of actin polymerization.

PI 3-kinase is inhibited in vivo by wortmannin with an IC_{50} of 5 nM [5] (wortmannin also inhibits myosin light chain kinase [6] and mitogen-activated protein kinase [7], but this requires 40–200 × higher concentrations). Neutrophil pretreatment with wortmannin and certain analogs causes sinusoidal oscillations in F-actin content and whole-cell light scattering having a period of 8–10 s [8,9]. The scattering oscillations apparently arise from cellular shape changes reflecting periodic actin polymerization-driven protrusion and retraction of weakly scattering lamellipods [8,10] and probably constitute a fundamental cellular response related to the amoeboid-like crawling motions of stimulated neutrophils [9].

Agonist-induced PI 3-kinase activity is coupled with stimulation of the small G protein Rho in Swiss 3T3 cells [11] as well as in human platelets, where Rho associates with the cytoskeleton upon stimulation [12,13]. Microinjection of Rho causes formation of actin stress fibers emanating from focal adhesions in Swiss 3T3 [14] and Vero cells [15], but the response is prevented by ADP-ribosylation of endogenous Rho. We investigated whether Rho plays a role in the F-actin-coupled oscillations in human neutrophil shape described earlier [8]. Intracellular Rho was inhibited by means of a genetically engineered chimeric toxin C3B [15] containing a membrane-translocating subunit derived from diphtheria toxin and a functional subunit (exoenzyme C3 from *Clostridium botulinum*) which selectively ADP-ribosylates and inactivates RhoA, B and C [15–17].

2. Experimental

2.1. Materials

[^{32}P]NAD (30 Ci/mmol) was purchased from NEN-Du Pont de Nemours, Dreieich, Germany; all other chemicals were obtained as described [9]. Neutrophils were isolated from buffy coats of single donors and suspended in test buffer [9].

2.2. ADP-ribosylation

Chimeric toxin C3B was prepared by genetic fusion of the exoenzyme C3 gene (coding for the catalytic subunit) to DNA coding for diphtheria toxin (the membrane-translocating subunit) [15]. Neutrophils ($1–10 \times 10^7$/ml) were incubated with C3B (4 μg/ml, ~0.1 μM) for 100 min at 37°C.

In vitro ADP-ribosylation was performed with postnuclear lysates [15]. Eight million cells incubated with or without C3B were washed once with ice-cold test buffer, resuspended in 350 μl ice-cold lysis buffer (2 mM $MgCl_2$/0.1 mM phenyl-methyl-sulfonyl-fluoride/10 μg/ml leupeptine/25 mM triethanolamine-HCl, pH 7.5), sonicated on ice using a probe sonicator and five 15-s pulses (Sonifier, Cell Disruptor B-30, Branson Sonic Power, Danbury, CT) and centrifuged for 10 min at 2000 × *g* and 4°C. The supernatant was used as postnuclear lysate: an aliquot containing 10 μg protein (determined using the protein assay reagent kit from Pierce, Rockford, IL) was incubated with 2.5 μM [^{32}P]NAD (20,000 dpm/pmol), 2.5 mM AMP and C3B (4 μg/ml) for 30 min at 37°C. The reaction was stopped by adding 5 μl sample buffer and incubating for 3 min at 90°C. An SDS-PAGE was performed, the gel was stained with Coomassie blue, dried and exposed in a phosphorimager (Molecular Dynamics, Sevenoaks, UK). A broad range protein standard (Bio-Rad, Hercules, CA) was used.

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Abbreviations: F-actin, filamentous actin; fMLP, *N*-formyl-Met-Leu-Phe; HWT, 17-hydroxywortmannin; PI 3-kinase, phosphatidylinositol 3-kinase.

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2.3. Measurements of F-actin and shape change

Total F-actin was determined using the method of Howard and Oresajo [18]. One million neutrophils were incubated for 100 min at 37°C in the presence or absence of C3B, stimulated with 10 nM fMLP for 10 s, fixed for 15 min at 37°C with 3.7% formaldehyde supplemented with 2 mM EDTA (to prevent cellular aggregation) and then permeabilized (67 μg lysophosphatidylcholine/ml) and stained with 0.33 μM Rhodamine phalloidin for 15 min at room temperature. Five thousand cells/sample were analysed in a Coulter Epics Profile II flow cytometer (Coulter Corporation, Hialeah, FL).

Neutrophil shape changes were assayed in suspensions of 8×10^5 cells/ml using laser turbidimetry as described [9].

3. Results and discussion

3.1. ADP-ribosylation of Rho by C3B

To examine whether the chimeric toxin C3B had entered the neutrophils and effectively ADP-ribosylated cellular Rho, in vitro ADP-ribosylation using [^{32}P]NAD was carried out on postnuclear lysates of untreated or C3B-pretreated neutrophils. Thereby, all C3B substrate which had not been previously ADP-ribosylated in vivo (especially in untreated control cells) was radiolabelled in vitro and could thus be autoradiographically assayed. Postnuclear lysate of cells which had been pretreated with C3B (4 μg/ml) for 100 min at 37°C contained no detectable substrate available for ADP-ribosylation with [^{32}P]NAD (Fig. 1, lane 1), whereas control lysate (from cells incubated with test buffer at 37°C) yielded a [^{32}P]ADP-ribosylated band at M_r ~23 kDa (Fig. 1, lane 2) corresponding to the previously reported M_r of exoenzyme C3 substrate [11,12,17,19]. These results indicate that endogenous Rho is effectively inactivated in vivo by C3B under the conditions we used.

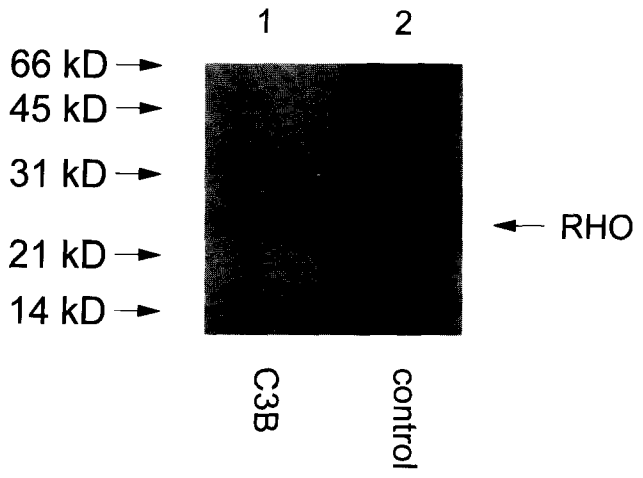

Fig. 1. The chimeric toxin C3B ADP-ribosylates Rho within neutrophils. Postnuclear lysates of cells treated with (lane 1) or without (control, lane 2) C3B were subjected to in vitro ADP-ribosylation in the presence of [^{32}P]NAD. SDS-PAGE of the lysates was performed and the gel was dried and analysed using a phosphorimager. The arrows show the position of the marker proteins stained with Coomassie blue in the same gel. Data of one experiment are shown; similar results were obtained in two experiments using neutrophils from different donors.

3.2. Actin polymerization and shape changes

Inactivation of Rho by C3B-mediated ADP-ribosylation did not inhibit the fMLP-induced actin polymerization sampled 10 s following stimulus addition (Fig. 2) or alter the time-dependent shape-sensitive scattering changes (Fig. 3). The results with F-actin are similar to those obtained by Koch et al. [20], who found that electropermeabilization of exoenzyme C3-like transferase from *Clostridium limosum* into HL-60 cells ADP-ribosylated Rho without inhibiting fMLP-induced actin polymerization.

Neutrophil shape oscillations dying out 40–60 s after agonist addition are routinely enabled by specific manipulations which inhibit PI 3-kinase activity, i.e. pretreatment with 1 μM 17-hydroxywortmannin (HWT) or wortmannin [5,8,9] or prestimulation with an agonist [9,21]. Activation of certain PI 3-kinases is affected by ADP-ribosylation of Rho in other cell types [11–13]. Therefore, we speculated that Rho inhibtion could also enable neutrophil oscillations. Fig. 3 shows that ADP-ribosylation of Rho by itself is not sufficient to enable fMLP-provoked oscillations. However, when treatment with C3B is used in conjunction with HWT, the oscillations are markedly enhanced (Fig. 4, Table 1) under conditions where PI 3-kinase activity is completely prevented [5]. In HWT-preincubated cells, ADP-ribosylation of Rho-enhanced amplitude and duration of scattering-detected shape oscillations (Fig. 4); additionally, oscillation frequencies in these cells were higher than

Table
Light scattering oscillation frequencies of C3B-treated and control neutrophils stimulated with fMLP

Treatment	Oscillation frequency in Hz and significance P for:			
	Extinction	P	90° scattering	P
C3B	0.122 ± 0.014 (8)	–	0.122 ± 0.013 (8)	–
Control	0.106 ± 0.013 (7)	0.04	0.108 ± 0.010 (10)	0.02

Power spectral density estimates. Neutrophils treated in the presence or absence (control) of C3B for 100 min at 37°C and 10 min preincubated with 1 μM HWT were stimulated with 10 nM fMLP. Table values are mean ± S.D.; parentheses enclose the number of independent experiments. P is the probability that the oscillation frequency obtained in both treatments is the same (Student's t-test).

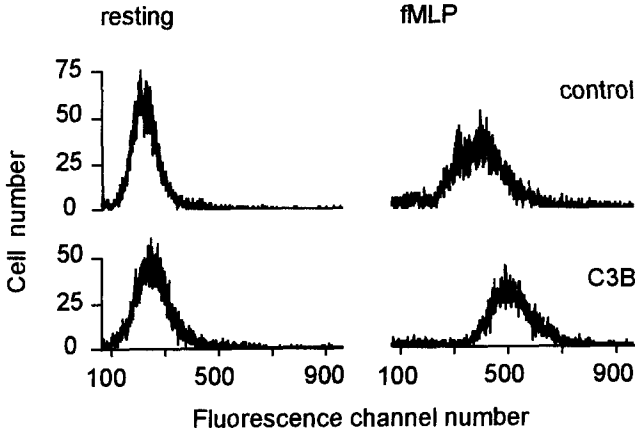

Fig. 2. Effect of Rho ADP-ribosylation on the basal and fMLP-stimulated F-actin content. Cells preincubated in the presence or absence of C3B were stimulated with fMLP (10 nM) for 10 s. Cells were fixed, stained with Rhodamine phalloidin and analysed for total F-actin in a flow cytometer. Data are expressed as fluorescence intensity/cell (fluorescence channel number) vs. cell number.

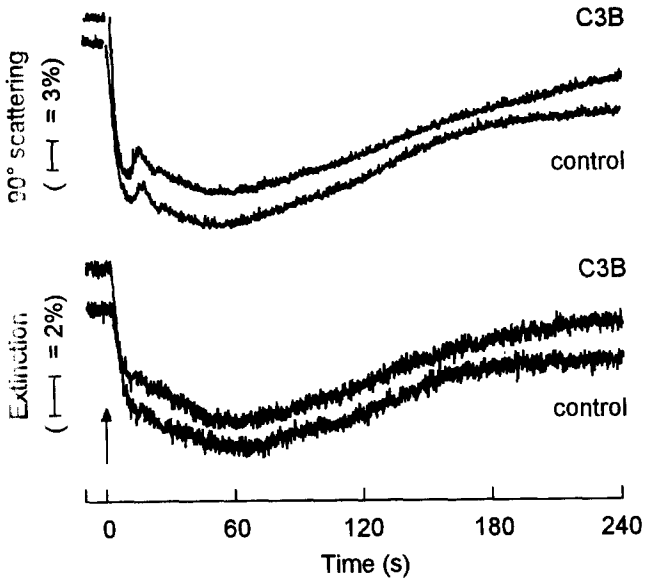

Fig. 3. Scattering-detected neutrophil shape changes in the presence and absence of Rho ADP-ribosylation (% of initial). Cells treated without or with C3B (100 min at 37°C) were stimulated with 10 nM fMLP at time 0 (arrow) and relative changes in 90° light scattering (top) and extinction (bottom) were measured. Similar results were found in four experiments using cells from different donors.

hose of control cells (Table 1). Thus, exposure of human neutrophils to HWT unmasks a pathway which regulates shape oscillations and is sensitive to modulation by Rho.

Agonist-induced changes in light scattering by suspended neutrophils reflect, in part, the generation of thin, veil-like lamellipods [8,10,22]. In contrast to morphological alterations in suspension, neutrophil migration over a substrate depends

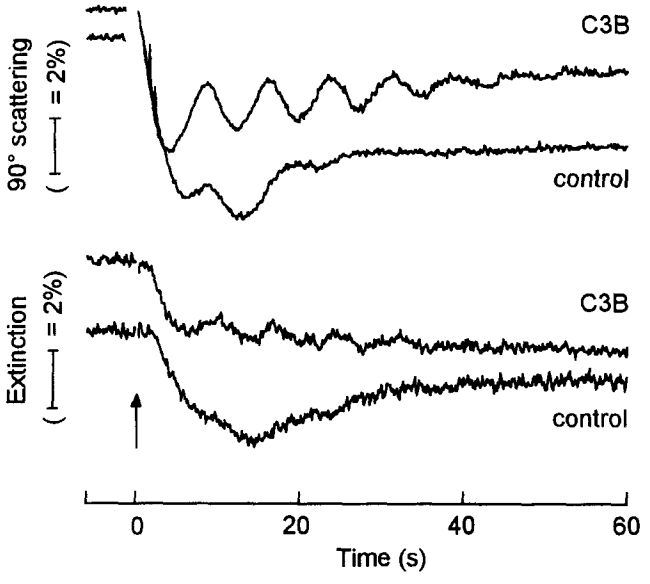

Fig. 4. Effect of Rho ADP-ribosylation on neutrophil shape oscillations. Neutrophils were pretreated with or without C3B for 100 min and with 1 μM HWT for 10 min; fMLP (10 nM) was added at time 0 (arrow) and relative changes in 90° light scattering (top) and extinction (bottom) were recorded. The data shown (% of initial) are representative for eight independent experiments.

upon reversible formation of adhesion sites and is blocked by exoenzyme C3-mediated ADP-ribosylation [19]. We suspect that Rho inhibtion prevents neutrophil translocation over a substrate by interfering with the formation of adhesion sites.

The finding that Rho inactivation enhances scattering-detected shape oscillations (in the presence of HWT) brings up questions about the regulatory mechanism involved in the oscillations. Existing evidence suggests that actin polymerization/depolymerization cycles are responsible for lamellipod formation and remodelling [8]. Two different F-actin pools exist in human neutrophils: a gelsolin-rich, labile F-actin pool and a stable F-actin pool [23,24]. Net F-actin growth induced by fMLP results from simultaneous addition of actin monomers and labile F-actin to the stable F-actin pool [25]. It is tempting to speculate that blocking Rho prior to stimulation partially shifts stable F-actin into the labile F-actin pool, thus enhancing agonist-induced actin polymerization and lamellipod formation. Time-resolved measurements of the labile F-actin and globular actin pools in C3B-treated cells, which could clarify this speculation, are not yet available. However, in HL-60 cells Rho inhibition decreases resting cell F-actin and enhances F-actin polymerization stimulated by fMLP in agreement with this hypothesis [20].

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