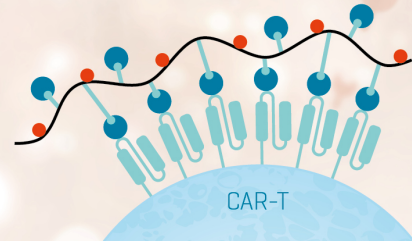


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J Immunol (2002) 169 (5): 2330–2336.

<https://doi.org/10.4049/jimmunol.169.5.2330>

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Requirement for RhoA Kinase Activation in Leukocyte De-Adhesion¹

Li Liu,^{2,3*} Barbara R. Schwartz,^{2*} Nancy Lin,^{*} Robert K. Winn,[†] and John M. Harlan^{*}

Leukocyte migration from bloodstream to tissue requires rapid coordinated regulation of integrin-dependent adhesion and de-adhesion. Whether de-adhesion is an active process mediated by a distinct signaling pathway(s) or a passive decay of initial adhesion remains undetermined. We found that blockade of RhoA with C3 exoenzyme or inhibition of RhoA kinase by the specific inhibitor Y-27632 enhanced phorbol ester-stimulated $\alpha_4\beta_1$ -dependent adhesion of Jurkat cells at 30 min. Similarly, Y-27632 treatment increased stimulated β_2 integrin-dependent neutrophil adhesion at 30 min but not at 5 min. Because reduced de-adhesion could mimic augmentation of adhesion at later time points, we developed an assay to measure de-adhesion specifically. Treatment of phorbol ester—or bacterial chemoattractant peptide—but not Mn^{2+} -stimulated neutrophils adherent to serum-coated plastic or endothelial cells with Y-27632 or C3 exoenzyme markedly reduced the rate of de-adhesion, while markedly increasing their spreading. RhoA kinase inhibitor effects on de-adhesion and spreading were reversed by treatment with the cytoskeletal-disrupting agent cytochalasin D. Treatment with Y-27632 influenced neither integrin activation epitope nor integrin clustering. We conclude that activation of RhoA kinase promotes leukocyte de-adhesion by inhibiting cytoskeletal-dependent spreading, and that these effects of RhoA kinase constitute a new mechanism for regulation of integrin receptor avidity. *The Journal of Immunology*, 2002, 169: 2330–2336.

Integrin-dependent leukocyte adhesion to endothelium and extracellular matrix components is critical for leukocyte recruitment to extravascular sites of inflammation and immune reaction (1, 2). Rapidly modulated de-adhesion is equally important for leukocyte emigration from bloodstream to tissue. A persistent increase in integrin affinity or avidity impairs leukocyte migration by freezing the receptor to ligand(s) (3, 4). Considerable progress has been made in elucidating the signaling pathways that modulate integrin affinity or avidity (5), but the intracellular signaling pathways involved in de-adhesion are largely unknown. The role of RhoA in integrin-mediated leukocyte adhesion is controversial. Blockade of RhoA function by C3 exoenzyme has been reported to inhibit β_2 integrin-dependent stimulated neutrophil adhesion (6). However, in other studies treatment with C3 exoenzyme did not inhibit β_2 integrin-dependent adhesion of neutrophils (7) or JY lymphocytic cells (8) or β_1 integrin-dependent adhesion of peripheral blood T cells (9) or U937 monocytic cells (10). Cultured human T lymphocytic cells adhering via β_1 integrin and freshly prepared human polymorphonuclear leukocytes (neutrophils), which undergo rapid β_2 integrin-dependent adhesion and de-adhesion when stimulated, were used to study the intracellular mechanisms regulating these events. We focused on RhoA and RhoA kinase, the downstream kinase of RhoA, which has been shown to mediate many RhoA functions (11–14). We were able to distinguish between effects on adhesion and de-adhesion by de-

signing an assay to specifically measure de-adhesion of adherent leukocytes using a rapidly penetrating and fast-acting reagent that could be added after removal of stimulus and nonadherent cells.

Materials and Methods

Cell culture

Human T lymphocytic Jurkat cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (BioWhittaker, Walkersville, MD) with 10% FBS (HyClone Laboratories, Logan, UT). HUVEC were isolated and cultured as previously described (15). They were grown in RPMI 1640 with the addition of 2 mM glutamine, sodium pyruvate, non-essential amino acids, 10 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin, 250 ng/ml fungizone (BioWhittaker), 90 μ g/ml heparin (Sigma-Aldrich, St. Louis, MO), bovine hypothalamic extract (gift of E. Raines, University of Washington, Seattle, WA), 10% bovine calf serum and 10% bovine calf serum supplemented with iron (HyClone Laboratories). For experiments, they were cultured in cell culture microwell plates (Nalge Nunc International, Naperville, IL).

Preparation of neutrophils from peripheral blood

Peripheral blood was obtained from normal donors with informed consent according to procedures approved by the Human Subjects Review Committee of the University of Washington. Blood was drawn into heparinized syringes and neutrophils were isolated using Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, followed by lysis of RBC (0.155 M NH_4Cl , 0.1 M $KHCO_3$, 1 mM EDTA, pH 7.4, at 4°C) and washing in RPMI containing 2% FBS.

Adhesion and de-adhesion assays

Jurkat cells were incubated overnight with C3 exoenzyme from *Clostridium botulinum* (Calbiochem, La Jolla, CA) at 37°C. Leukocytes were labeled with 5 μ M calcein-AM (Molecular Probes, Eugene, OR) at room temperature for 45 min and washed. Neutrophils were treated with C3 exoenzyme for 4 h, with calcein-AM added for the last 15 min. For inhibition of RhoA kinase, calcein-labeled leukocytes were treated with indicated concentrations of RhoA kinase inhibitor Y-27632 (Ref. 16; a gift of Welfide Corporation, Osaka, Japan) for 10 min. Jurkat cells (5×10^4 /well) were stimulated with PMA (Sigma-Aldrich) or with mAb 8A2 (17, 18) to adhere to fibronectin (1 μ g/ml; Life Technologies, Rockville, MD) for 30 min at 37°C. Neutrophils (1×10^5 /well) were stimulated with phorbol

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Received for publication January 25, 2002. Accepted for publication June 26, 2002.

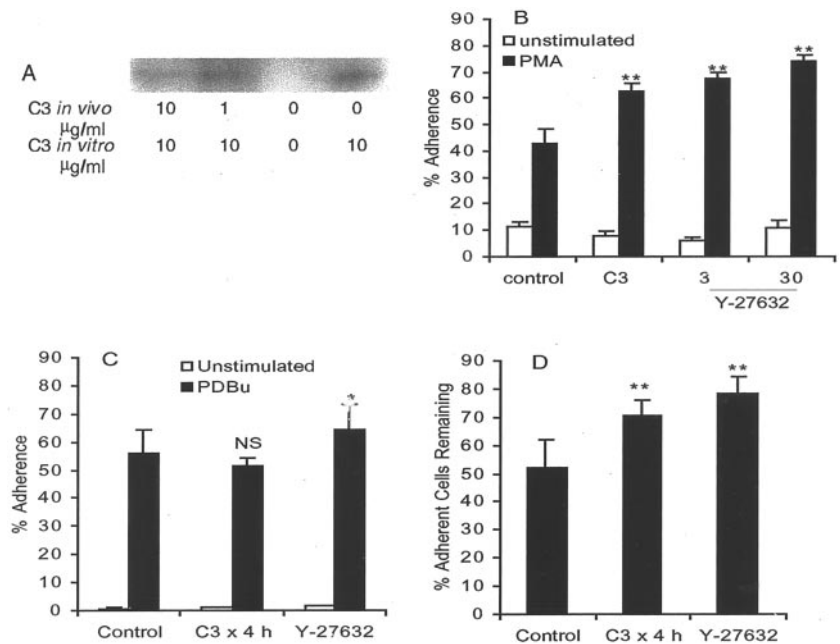
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¹This work was supported by U.S. Public Health Service Grants HL18645 and HL30542 (to J.M.H.) and a Parker B. Francis Fellowship (to L.L.).

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FIGURE 1. Blockade of RhoA or RhoA kinase activity does not inhibit phorbol ester-stimulated leukocyte adhesion. **A**, Jurkat cells were treated overnight with 0, 1 or 10 $\mu\text{g/ml}$ C3 exoenzyme and lysed. The lysates were then treated with 0 or 10 $\mu\text{g/ml}$ C3 exoenzyme and [^{32}P]NAD. Treatment with C3 exoenzyme *in vivo* significantly reduced the amount of RhoA protein that could be labeled with [^{32}P] *in vitro*. **B**, Jurkat cells treated overnight with 10 $\mu\text{g/ml}$ C3 exoenzyme or for 1 h with 3 or 30 μM Y-27632 were allowed to adhere to fibronectin for 30 min in the absence or presence of phorbol ester. Values are means \pm SD of four replicates in a representative experiment. **C** and **D**, Neutrophils were treated with buffer, 20 $\mu\text{g/ml}$ C3 exoenzyme, or 10 μM Y-27632 for 4 h. Neutrophils were allowed to adhere to serum-blocked plastic for 30 min in the absence or presence of PDBu (**C**), and the adherent cells were allowed to de-adhere for 30 min in the absence of stimulus (**D**). Y-27632 was added to Y-27632-pretreated cells for the de-adhesion. Values are means \pm SD of 10 replicates. *, $p < 0.05$; **, $p < 0.01$, compared with control.



12,13-dibutyrate (PDBu)⁴ (Calbiochem), fMLP (Sigma-Aldrich), or Mn²⁺ (MnCl₂; Sigma-Aldrich) to adhere to serum-blocked cell culture microwell plates or to confluent HUVEC at 37°C. One-tenth volume of 10% methanol-free formaldehyde (Polysciences, Warrington, PA) was added to fix the cells at times indicated. Percent adherence was calculated by measuring the fluorescence in each well of the total cells, and of the adherent cells after two washes, in a Cytofluor 4000 fluorescence plate reader (PerSeptive Biosystems, Framingham, MA). To measure de-adhesion, neutrophils were stimulated to adhere to serum-blocked plastic or to HUVEC, after which nonadherent cells were decanted and the plates were washed twice. Medium with or without 10 μM Y-27632 was then added and the plates were incubated at 37°C. Cytochalasin D (Sigma-Aldrich) was added 10 min after the Y-27632. At the indicated times, the wells were fixed, and percent neutrophils remaining adherent was determined at the end by measuring fluorescence before and after additional washes. MnCl₂ quenched calcein fluorescence, even as it induced strong adhesion. Consequently, staining with crystal violet was performed at the end to confirm relative numbers of cells. After fluorescence readings on neutrophil adherence to confirm the even loading of cells, washed plates were read again and stained with 0.5% crystal violet (Sigma-Aldrich) in 20% methanol, extensively washed in water, and stain solubilized with 0.1% sodium citrate in 50% ethanol. The absorbance at 570 nm (reference 650 nm) was read on a Titertek Multiscan MCC/340 plate reader (Flow Laboratories, McLean, VA).

ADP-ribosylation assay of RhoA

Jurkat cells (2×10^6) were treated with 10 $\mu\text{g/ml}$ C3 exoenzyme as indicated. After overnight incubation, the cells were washed with cold PBS, harvested in 250 μl of cold lysis buffer, 50 mM HEPES, pH 7.4, containing CØmplete Mini EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN) and sonicated. Aliquots (100 μg of protein) of the lysate were incubated with [^{32}P]NAD (American Radiolabeled Chemicals, St. Louis, MO) and 10 μg of C3 exoenzyme at 37°C for 30 min before Laemmli buffer was added. The samples were heated for 3 min at 95°C and run on 12% SDS-PAGE. [^{32}P]ADP-ribosylated proteins were detected by autoradiography with a PhosphorImager from Molecular Dynamics (Krefeld, Germany).

Confocal microscopy

Neutrophils in RPMI 1640 plus 5% heat-inactivated adult bovine serum (HyClone) were stimulated with 1 μM fMLP for 10–15 min at 37°C for cells to adhere on serum-blocked eight-well Lab-Tek chamber slides. fMLP and nonadherent cells were removed by three washes with medium. The adherent cells were either fixed immediately or incubated at 37°C for 15 min in the presence or absence of 15 μM Y-27632 and then fixed. Integrin CD11b was stained with mAb OKM1 (ATCC CRL 8026) purified

from mouse ascites followed by FITC-goat anti-mouse F(ab')₂, human-adsorbed second Ab (Caltag Laboratories, So. San Francisco, CA). Photomicrographs were made with a Bio-Rad Radiance 2000 confocal microscope (Hercules, CA) equipped with a krypton-argon laser and a Nikon inverted microscope (Tokyo, Japan).

Neutrophil spreading

Neutrophils in RPMI with 2% FBS were incubated with or without 10 μM Y-27632 for 30 min and then adhered to serum-blocked eight-well Lab-Tek chamber slides (Nalge Nunc International) with or without 10 μM fMLP in the presence or absence of 0.25 $\mu\text{g/ml}$ cytochalasin D. Cells were incubated at room temperature for 30 min before fixing with 0.5% formaldehyde. After three washes to remove nonadherent cells, actin was stained with rhodamine-phalloidin and nuclei with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Photomicrographs were made on a Nikon Eclipse E800 Fluorescent microscope, using a 60 \times 0.95 Nikon dry objective lens and filters to detect DAPI and rhodamine fluorescence above 590 nm. Areas were determined on a Nikon Diaphot 200 microscope using NIH Image Scion 1.6 software. At least 150 cells were measured per sample.

Flow cytometry

Neutrophils were suspended in RPMI containing 5% heat-inactivated adult bovine serum and were incubated with or without 30 μM Y-27632 before activation at 37°C for 15 min with fMLP in the presence of Alexa-488 mAb 327C, which recognizes a β_2 integrin activation epitope (Ref. 17; the generous gift of D. Staunton, ICOS Corporation, Bothell, WA). To measure the effect of Y-27632 on disappearance of the active epitope, neutrophils that had bound mAb 327C were washed to remove stimulant and unbound mAb and incubated with or without Y-27632 at 37°C for 30 min. Samples were fixed with 1% formaldehyde, data were acquired on a BD Biosciences FACScan flow cytometer (Mountain View, CA) and were analyzed using WinMDI (J. Trotter, Scripps Institute, La Jolla, CA). Data were normalized by dividing mean fluorescences in each experiment by those obtained with mAb 5D1, which recognizes the β_1 integrin subunit, not expressed by neutrophils under normal circumstances.

Significance was determined with the two-tailed Student's *t* test with unequal variance, using Microsoft Excel (Redmond, WA).

Results

Blockade of RhoA or RhoA kinase does not inhibit PMA-stimulated β_1 integrin-dependent Jurkat cell adhesion

Overnight treatment of Jurkat cells with C3 exoenzyme significantly reduced the amount of RhoA protein that could be labeled with ^{32}P *in vitro*, demonstrating that the C3 exoenzyme had penetrated the intact cells and ADP-ribosylated RhoA *in vivo* (Fig. 1A,

⁴ Abbreviations used in this paper: PDBu, phorbol 12,13-dibutyrate; DAPI, 4',6-diamidino-2-phenylindole.

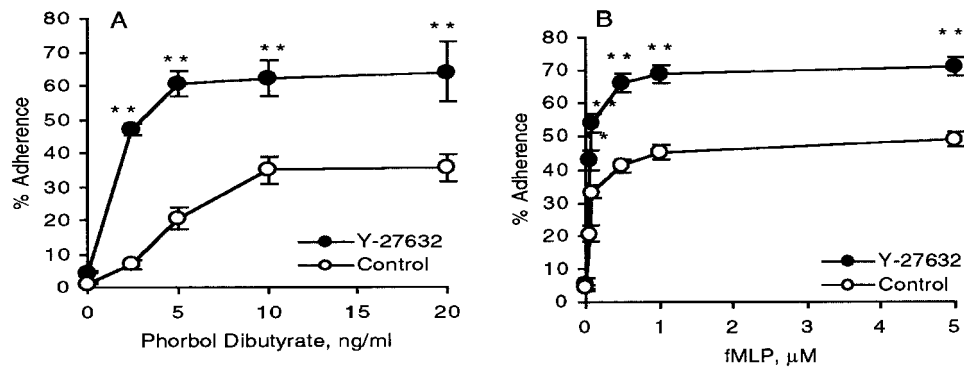


FIGURE 2. Inhibition of RhoA kinase enhances neutrophil adhesion. Calcein-labeled neutrophils were stimulated to adhere to serum-blocked plastic for 30 min. *A*, PDBu, *B*, fMLP. Values are means \pm SD of five replicates in a representative experiment. **, $p < 0.01$.

lanes 1 and 2; compare to lane 4). In the absence of C3 exoenzyme in vitro there was no RhoA ADP-ribosylation (Fig. 1A, lane 3). However, treatment with a concentration of C3 exoenzyme which ADP-ribosylated most of the RhoA protein in the cells did not inhibit β_1 integrin-dependent PMA-stimulated adhesion to fibronectin (Fig. 1B). This PMA-stimulated adhesion was inhibited by mAbs to α_4 and β_1 integrin subunits (data not shown). Furthermore, the specific RhoA kinase inhibitor Y-27632 did not inhibit Jurkat cell adherence to fibronectin (Fig. 1B). In fact, inhibition of the RhoA/RhoA kinase pathway appeared to increase

PMA-stimulated Jurkat cell adhesion, but had no effect on adhesion stimulated by mAb 8A2, which directly increases β_1 integrin affinity (Refs. 18 and 19; data not shown).

Blockade of Rho or RhoA kinase inhibits β_2 integrin-dependent adherent neutrophil de-adhesion

Neutrophils pretreated with C3 exoenzyme and stimulated to adhere to serum-blocked plastic evidenced adhesion similar to control (Fig. 1C), but less de-adhesion than control (Fig. 1D). Similarly, in the presence of Y-27632, neutrophils adhered more and

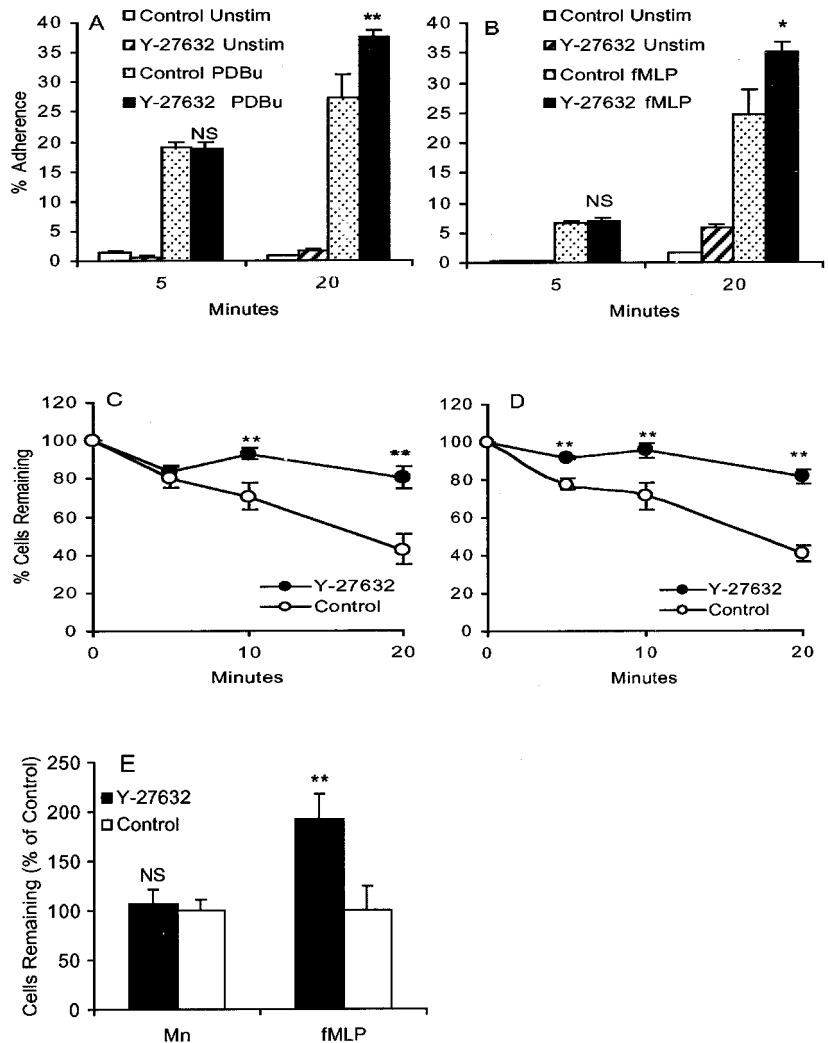


FIGURE 3. Inhibition of RhoA kinase does not augment initial neutrophil adhesion, but does inhibit fMLP- and PDBu-stimulated neutrophil de-adhesion. *A* and *B*, Adhesion assay. The time-dependent adhesion of untreated or Y-27632-treated (10 μ M) neutrophils to serum-blocked plastic was determined. Cells were fixed at 5 or 20 min after stimulation with 20 ng/ml PDBu (*A*) or 10 μ M fMLP (*B*) and percent adherence was determined. Values are means \pm SD of four replicates in a representative experiment. *C* and *D*, De-adhesion assay. Neutrophils were stimulated to adhere to serum-blocked plastic using either 20 ng/ml PDBu (*C*) or 10 μ M fMLP (*D*). After total neutrophils per well were determined, nonadherent cells were decanted and the plates were washed twice. Medium with or without 10 μ M Y-27632 was then added and the plates were incubated at 37°C. At the indicated times the cells were fixed, the wells were washed and the percentage of neutrophils remaining adherent was determined. Values are means \pm SD of five replicates in a representative experiment. *E*, Crystal violet staining of Mn^{2+} - or fMLP-adherent neutrophils after 30-min de-adhesion, as percent of untreated control. *, $p < 0.05$; **, $p < 0.01$, compared with control.

de-adhered less than controls (Fig. 1, *C* and *D*). Stimulation of neutrophils with the chemoattractant fMLP or the protein kinase C activator PDBu produced a marked, time-dependent increase in adhesion to serum-coated plastic, and this adhesion was totally inhibited by the blocking CD18 mAb 60.3 (data not shown). Inhibition of RhoA kinase with Y-27632 enhanced both fMLP- and PDBu-stimulated neutrophil adhesion at 30 min (Fig. 2). However, further investigation of the time course of adhesion showed that Y-27632 had no effect on fMLP- or PDBu-stimulated neutrophil adhesion at 5 min, despite a 30-min pretreatment, but significantly enhanced adhesion at 20 min (Fig. 3, *A* and *B*). The lack of effect on early adhesion, despite preincubation with the inhibitor, but apparent augmentation of adhesion at later time points suggested that the inhibitor might actually be reducing de-adhesion of the adherent cells. In support of this, the effect of C3 exoenzyme implicated RhoA specifically in neutrophil de-adhesion. To separate the process of de-adhesion from that of adhesion, it was necessary first to stimulate neutrophils to adhere using reagents that could be removed, and second to administer to the adherent cell reagent(s) that penetrate easily and act quickly. fMLP and PDBu met the first requirement, and the RhoA kinase inhibitor, Y-27632, met the second. Therefore, we examined the participation of RhoA in neutrophil de-adhesion using stimulated neutrophils and Y-27632.

Neutrophils were stimulated to adhere. After a 15-min period of initial adhesion, nonadherent cells and stimuli were removed, and the adherent cells were incubated with control medium or medium containing Y-27632. Cells remaining adherent at various time points were measured. The rate of de-adhesion was markedly lower in cells treated with the RhoA kinase inhibitor Y-27632, indicating that RhoA activation promotes de-adhesion (Fig. 3, *C* and *D*). Y-27632 had no effect on neutrophils that had been stimulated to adhere using Mn^{2+} to augment β_2 integrin affinity directly (Fig. 3*E*). The induction and decay of an activation epitope of β_2 integrin was investigated using mAb 327C (17). Y-27632 treatment had no effect on either induction (Fig. 4*A*) or disappearance (Fig. 4*B*) of activation epitope. Staining for integrin with anti-CD11b mAb did not demonstrate clear changes in integrin clustering (Fig. 4*C*). Adherent neutrophils incubated with Y-27632 after removal of stimulus and nonadherent cells exhibited a more spread morphology than those incubated in its absence. CD11b integrin was distributed in a much larger area in the presence of Y-27632 (Fig. 4*C*).

We next determined whether blockade of RhoA kinase had the same effect on stimulated neutrophil adhesion to and de-adhesion from endothelial cells. Treatment of neutrophils with Y-27632 augmented fMLP-stimulated neutrophil adhesion to HUVEC (Fig. 5*A*). This effect was not caused by carryover of a trace amount of inhibitor from neutrophil to HUVEC, because fMLP-stimulated neutrophils adhered less well to HUVEC pretreated with Y-27632 (Fig. 5*B*), consistent with the proposed role of endothelial RhoA in integrin-ligand clustering (20). Treatment with Y-27632 did reduce the rate of neutrophil de-adhesion from HUVEC (Fig. 5, *C* and *D*). Y-27632 also slowed de-adhesion of MO7E from Chinese hamster ovary cells transfected with VCAM-1, a β_1 integrin-dependent process (data not shown).

Inhibition of RhoA kinase promotes neutrophil spreading

Previous studies had shown that inhibition of RhoA function by treatment with C3 exoenzyme promoted monocyte spreading (21). Consistent with this result, we found that blockade of RhoA kinase by Y-27632 dramatically increased neutrophil spreading on serum-coated plastic (Fig. 6; Table I). The average area of Y-27632-

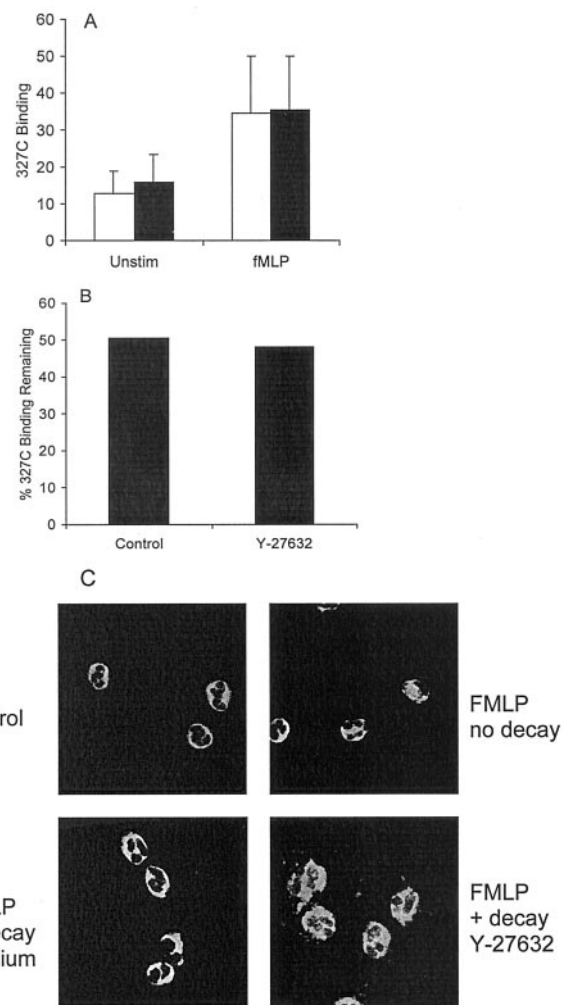
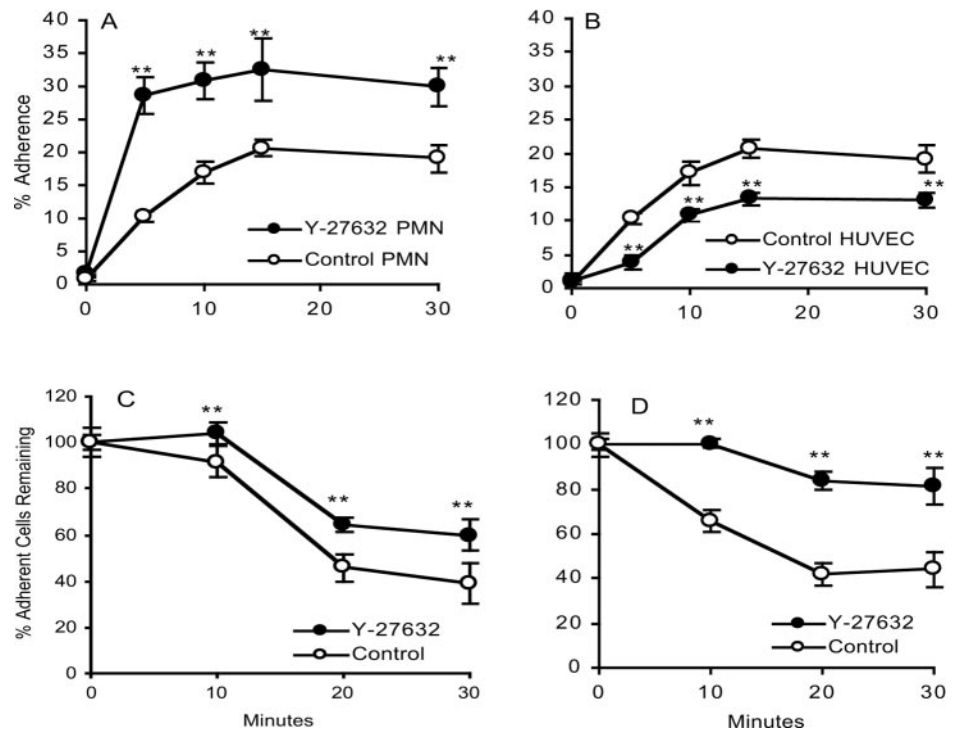


FIGURE 4. Y-27632 does not influence stimulation of or decay of expression of CD18 activation epitope and clustering in neutrophils stimulated by fMLP. Binding of mAb 327C to neutrophils was measured by flow cytometry. *A*, Neutrophils were preincubated with or without Y-27632 and then were stimulated with fMLP at 37°C for 15 min in the presence of Alexa-conjugated mAb 327C. Mean fluorescences were divided by those of mAb 5D1 (anti- β_1 integrin) to normalize data between experiments. □, control; ■, Y-27632. Data are the means \pm SEM of 327C binding from four experiments (NS). *B*, Neutrophils that had been stimulated with fMLP in the presence of Alexa-mAb 327C were washed and incubated at 37°C for 30 min in the presence or absence of Y-27632 to measure effect of the RhoA kinase inhibitor on decay of expression. *C*, Neutrophils were allowed to adhere (control) or were stimulated with fMLP to adhere to serum-blocked chamber coverslips for 15 min at 37°C. fMLP and nonadherent cells were removed by three washes with medium. The adherent cells were either fixed immediately (no decay) or incubated at 37°C for 15 min in the presence (fMLP, decay + Y27632) or absence (fMLP, decay in medium) of Y-27632. Neutrophils were stained with mAb OKM1 for CD11b. Photomicrographs were made with a confocal microscope (Bio-Rad Radiance 2000).

treated, fMLP-stimulated cells was 1.8 times that of control neutrophils. Because spreading requires a reorganization of the actin cytoskeleton, we tested the effect of cytochalasin D, which disrupts actin filament reorganization and thereby prevents cell spreading. Cytochalasin D completely blocked the increased neutrophil spreading induced by Y-27632 (Fig. 6; Table I), and neutrophils co-incubated with cytochalasin D and Y-27632 de-adhered at a rate similar to untreated cells (Fig. 7).

FIGURE 5. Inhibition of neutrophil RhoA kinase augments stimulated neutrophil adhesion to, and reduces neutrophil de-adhesion from, endothelial cells. *A* and *B*, Adhesion assay. Neutrophils or HUVEC were treated for 1 h with 10 μ M Y-27632 and then washed. fMLP-stimulated adherence of control or Y-27632-treated neutrophils to untreated HUVEC (*A*) or untreated neutrophils to control or Y-27632-treated HUVEC (*B*) was determined. Values are means \pm SD of six replicates in a representative experiment. *C* and *D*, De-adhesion assay. Neutrophils were stimulated to adhere to HUVEC with 20 ng/ml PDBu (*C*) or 10 μ M fMLP (*D*). After total neutrophils per well were determined, nonadherent cells were decanted and the plates were washed twice. Medium with or without 10 μ M Y-27632 was then added and the plates were incubated at 37°C. At the indicated times, the cells were fixed, the wells were washed, and percent neutrophils remaining adherent was determined. **, $p < 0.01$.



Discussion

In this study, we report a role for the RhoA/RhoA kinase pathway in β_1 and β_2 integrin-dependent leukocyte adhesion and de-adhesion. We found that blockade of RhoA did not inhibit $\alpha_4\beta_1$ integrin-mediated adhesion of Jurkat T lymphocytes to fibronectin. When assayed at 30 min, PMA-stimulated, but not mAb 8A2-stimulated, adhesion of Jurkat cells treated overnight with C3 exoenzyme or treated with the specific RhoA kinase inhibitor Y-27632 was apparently enhanced. Similarly, neutrophils treated with Y-27632 evidenced greater β_2 integrin-dependent adhesion. A careful analysis of the time course of stimulated neutrophil adhesion showed that the Rho kinase inhibitor had no effect on early adhesion at 5 min, but significantly augmented adhesion at 20 and

30 min. We considered three possible explanations for the effect on late but not early adhesion: 1) drug penetration dynamically controls the process; 2) the inhibitor is a slow-acting drug; 3) the effect is on the process of de-adhesion rather than on adhesion. Regardless of whether cells were preincubated with the inhibitor for 30 min, there was no effect on early adhesion. Therefore, it seemed most likely that the effect of the inhibitor was to reduce de-adhesion. The short life span of neutrophils *ex vivo* limits the use of approaches such as overexpression of constitutive active or dominant negative constructs, which after all do not distinguish between effects on initial adhesion and de-adhesion. We designed an assay to study de-adhesion of already adherent cells, taking advantage of the ability of Y-27632 to penetrate cells rapidly and act

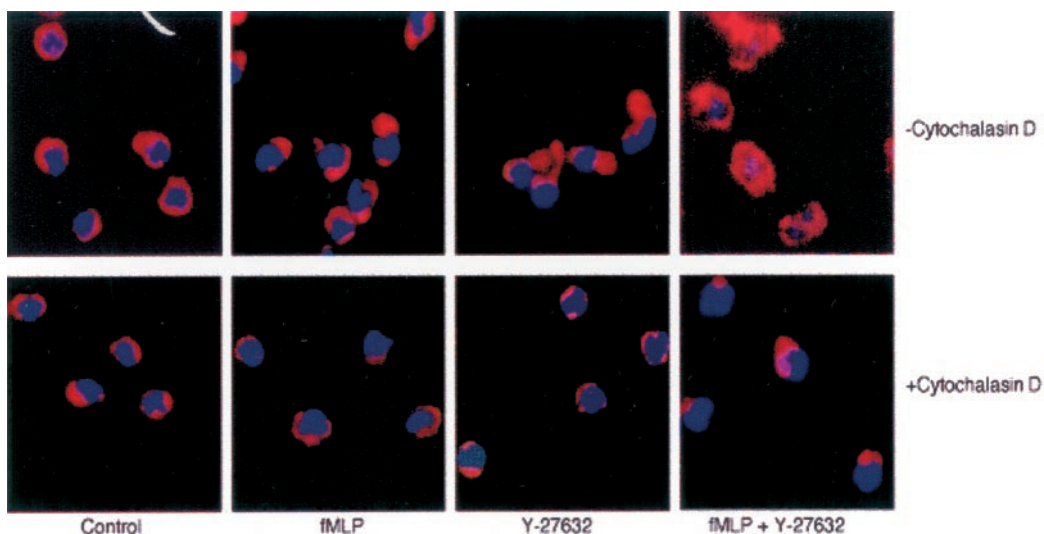


FIGURE 6. Inhibition of RhoA kinase induces spreading of stimulated neutrophils. Neutrophils were incubated with or without 10 μ M Y-27632 for 30 min and then allowed to adhere to a serum-blocked eight-chamber slide with or without 10 μ M fMLP in the absence (–Cytochalasin D) or presence (+Cytochalasin D) of 0.25 μ g/ml cytochalasin D. Cells were incubated at room temperature for 30 min before fixing and staining with rhodamine-phalloidin (red) for filamentous actin and DAPI (blue) for nuclei. Photomicrographs were made with a Nikon Eclipse E800 fluorescent microscope.

Table I. Spreading of neutrophils stimulated with fMLP in the presence of Y-27632 was abolished by cytochalasin D^a

	Unstimulated	fMLP	Y-27632	Y-27632 + fMLP
Control	1.0	1.1	1.0	1.8**
Cytochalasin D	1.1	1.0	1.0	1.1

^a The areas of neutrophils treated as in Fig. 5 were determined using NIH Image Scion 1.6. At least 150 cells in each sample were measured. The area of unstimulated control was set at 1.0. **, $p < 0.01$, compared to control.

quickly (22). Using this assay system, we observed that treatment with C3 exoenzyme or Y-27632 had more significant effects on the fraction of cells that de-adhered than they had had on the fraction of cells that had adhered in the same time frame (Fig. 1, C and D). This further suggested that RhoA and RhoA kinase were involved in leukocyte de-adhesion signal pathways.

Treatment of adherent neutrophils with Y-27632 significantly increased the fraction of cells remaining adherent (Fig. 3D). Differences could be observed within 5 min, confirming that the apparent increase in adhesion seen with Y-27632 was actually inhibition of de-adhesion and was not due to slow penetration of or slow activity by Y-27632 (Fig. 3B). The de-adhesion studies reported in this study focused on stimulated neutrophils, which adhere to serum-blocked plastic and to HUVEC entirely via β_2 integrins (data not shown). However, we obtained identical results with Y-27362 on β_1 integrin-dependent MO7E adhesion to and de-adhesion from VCAM-1-transfected Chinese hamster ovary

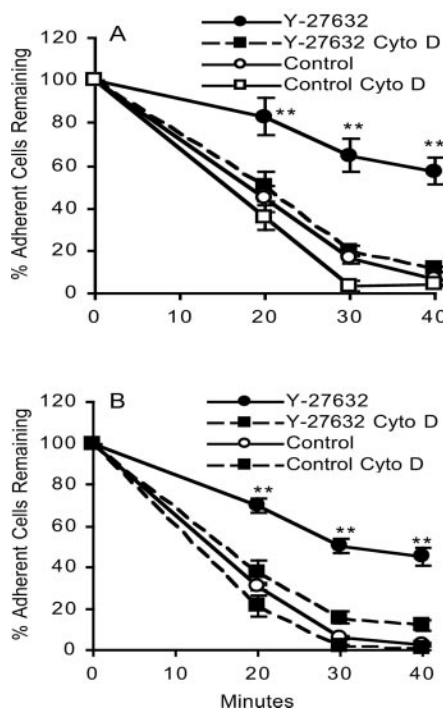


FIGURE 7. RhoA decreases cytoskeletal-dependent adhesion. Neutrophils were stimulated to adhere to serum-blocked plastic by a 15-min incubation with (A) 20 ng/ml PDBu or (B) 10 μ M fMLP. After washes to remove nonadherent neutrophils, remaining cells were treated with or without 10 μ M Y-27632 at 37°C for 10 min, and then 0.25 μ g/ml cytochalasin D or medium was added. Beginning 10 min later, wells were fixed at the time points indicated, and the percent adherence was determined. Values are the means \pm SD of four replicates in a representative experiment. **, $p < 0.01$, compared with control.

cells (data not shown), which argues that the role of Rho kinase in de-adhesion is not leukocyte type or integrin subunit-specific.

Mn²⁺ stimulates neutrophil adherence via affinity modulation (23), and de-adhesion of Mn²⁺-induced adherent neutrophils was insensitive to the Rho kinase inhibitor (Fig. 3E). Y-27632 also did not induce spreading of Mn²⁺-stimulated neutrophils (data not shown). Similarly, mAb 8A2-induced adherence of Jurkat cells was not enhanced by Y-27632 (data not shown). These results likely reflect differences in cytoskeletal involvement between affinity- and avidity-dependent mechanisms of cell adhesion (19, 24). Phorbol esters and fMLP, but not 8A2 and Mn²⁺, increase integrin mobility and clustering, and induce cell adhesion predominantly by modulation of avidity involving cytoskeletal reorganization (37). Thus, the effect of Y-27632 on phorbol ester- or fMLP-stimulated, but not on mAb 8A2- or Mn²⁺-stimulated, adherence is consistent with the involvement of the cytoskeleton in cell spreading. In further support of this is the failure of Y-27632 to influence either induction of the β_2 integrin activation epitope by fMLP (Fig. 4A) or its disappearance (Fig. 4B). There were no evident changes in clustering of integrin CD11b, but adherent cells washed and then incubated with Y-27632 were much more spread than those incubated without it (Fig. 4C). This, in turn, increased the number of integrin-ligand interactions, which would strengthen cell adhesion.

Aepfelbacher et al. (21) reported that blockade of RhoA by C3 exoenzyme caused monocyte spreading, suggesting that the RhoA signaling pathway promoted cell rounding. Similarly, we found that the RhoA kinase inhibitor Y-27632 markedly increased neutrophil spreading by 80% (Fig. 6; Table I). Although high doses of cytochalasin D inhibit adhesion, low doses increase adhesion by promoting integrin receptor diffusion and ligand-induced clustering (24, 25). Cytochalasin D, at a dose that did not inhibit adhesion of either control or Y-27632-treated fMLP-stimulated neutrophils (data not shown), increased de-adhesion of Y-27632-treated neutrophils (Fig. 7). When actin filament reorganization was prevented by cytochalasin D, Y-27632-treated cells remained round (Fig. 6). Even though cytochalasin D has been reported to activate RhoA in adherent cells (26), it is unlikely RhoA activation was responsible for the effect of cytochalasin D observed in this study because RhoA kinase is downstream of RhoA. It is more likely that cytochalasin D accelerated Y-27632-treated cell de-adhesion and abrogated cell spreading by interrupting the cytoskeleton network (24). It has been reported that Y-27632 can suppress myosin L chain phosphorylation (27). The effect of RhoA may result from RhoA kinase-mediated phosphorylation of myosin L chain (28) and the myosin-binding subunit of myosin L chain, as has been reported for neutrophils and multiple other cell types (27–33). Phosphorylation of myosin by RhoA kinase stimulates actin-activated ATPase activity of myosin II, whereas phosphorylation of myosin L chain phosphatase by RhoA kinase inhibits its activity, leading indirectly to increased myosin L chain phosphorylation (11). Both of these activities of RhoA kinase would thus promote actomyosin assembly and cell contraction or rounding. The RhoA kinase-mediated rounding would decrease cell spreading and thereby reduce interactions between neutrophil β_2 integrin receptors and cellular or extracellular ligands, promoting de-adhesion (Fig. 7). Conversely, increased spreading and a consequent increase in β_2 integrin-dependent avidity produced by blockade of RhoA kinase would impair neutrophil migration, as has been reported (27).

Anderson et al. (7) reported slower migration of neutrophils pretreated with C3 exoenzyme in a flow chamber model. Several recent studies have used time-lapse video microscopy to observe the effects of pretreatment with RhoA and RhoA kinase reagents

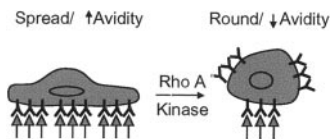


FIGURE 8. RhoA kinase promotes neutrophil de-adhesion by reducing cytoskeletal-dependent spreading. Cytoskeletal-dependent spreading of neutrophils enhances integrin-ligand interactions, thereby increasing avidity. Activation of RhoA kinase leads to actomyosin assembly and contraction with cell rounding, thus decreasing spreading and reducing avidity. Because high-avidity binding inhibits cell migration by “freezing” integrin receptors to ligand (4), RhoA kinase-mediated de-adhesion is necessary for optimal migration (27, 34).

on leukocyte migration. Worthylake et al. (34) reported that blockade of RhoA and RhoA kinase prevented monocyte tail retraction and transendothelial migration. Alblas et al. (35) found that RhoA and RhoA kinases were involved in eosinophil rear release in migration assays. Lymphocyte chemotaxis was abolished by pretreatment with C3 exoenzyme or Y-27632 (36). Our investigation of de-adhesion separately from adhesion extends the conclusions of these migration studies and confirms that the inability of cells pretreated with blockers of RhoA and RhoA kinase activity to migrate results specifically from impairment of de-adhesion.

In summary, blockade of RhoA kinase promoted cell spreading and impaired de-adhesion. By preventing spreading and promoting de-adhesion, activation of the RhoA/RhoA kinase pathway constitutes another mechanism to regulate integrin avidity (Fig. 8).

Acknowledgments

We thank Dr. Akiko Iwata for generous aid with the confocal microscope and Kristine Eiting for providing HUVEC.

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