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Role of the cytoskeleton in rapid activation of CD11b/CD18 function and its subsequent downregulation in neutrophils

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SUMMARY

When rolling adherent neutrophils are stimulated, they rapidly immobilise through activation of integrin CD11b/CD18, and then modulate attachment through this integrin to allow migration. We investigated links between cytoskeletal rearrangement and changes in function of integrin CD11b/CD18 in neutrophils stimulated with formyl peptide (fMLP). Neutrophils treated with the actin-polymerising agent jasplakinolide became rolling adherent on monolayers of activated platelets, but could not use CD11b/CD18 to become immobilised when fMLP was perfused over them. If treated with jasplakinolide after fMLP, the cells stopped migrating but could not detach when fMLP was removed. Jasplakinolide did not inhibit changes in intracellular Ca^{2+} seen after fMLP treatment, or inhibit neutrophil immobilisation induced by externally added Mn^{2+}. Thus cytoskeletal rearrangement was directly implicated in upregulation and, later, downregulation of CD11b/CD18 binding. Inhibition of RhoA with C3-transferase caused a dose-dependent reduction of initial rolling adhesion of neutrophils, and reduced the rate of migration after stimulation; however, neither the conversion of rolling to stationary adhesion, nor the ability of neutrophils to detach on removal of the stimulus, were inhibited. Thus, Rho may regulate actin polymerisation and motility in neutrophils, but did not appear to control integrin-mediated adhesion itself. Integrin binding may be promoted by disruption of links to the cytoskeleton, effected through depolymerisation of actin or cleavage of linking protein talin by calpain. Disruption of actin filaments with cytochalasin D did not, however, cause integrin-mediated immobilisation of rolling neutrophils. Although the calpain inhibitor calpeptin did inhibit the adhesion response to fMLP, this was only at doses where actin polymerisation was also ablated. We suggest that the cytoskeleton actively regulates binding conformation of CD11b/CD18 as well as its mobility in the membrane.

Key words: Neutrophil, Integrin, CD11b/CD18, Adhesion, Migration

INTRODUCTION

To exit the circulation, leukocytes typically use selectin receptors to form rolling attachments on the vessel wall, and then use β2-integrins to anchor firmly and support onward migration over the endothelial cells (Mackay and Imhof, 1993; Springer, 1995). The β2-integrins on unstimulated, circulating leukocytes have low affinity for ligand, so that rapid functional activation of integrins while rolling is required for precise localisation. To enable migration, binding must be downregulated again and new bonds formed on a time scale of minutes (Huttenlocher et al., 1996). Flowing neutrophils, for instance, can respond to surface-bound chemoattractants IL-8 and PAF in less than 1 second (Rainger et al., 1997), and within minutes of activation, migrate at a speed equivalent to about one cell length per minute (Rainger et al., 1997). The processes by which integrins alter their binding characteristics rapidly and reversibly remain poorly understood (for recent reviews, see e.g. Hughes and Pfaff, 1998; Williams and Solomkin, 1999). We chose to investigate the regulation of binding of the neutrophil β2-integrin CD11b/CD18, which has the ability to support both rapid immobilisation and migration (Hughes et al., 1992; Sheikh and Nash, 1996).

The cytoplasmic domain of the β2-integrin is linked to the cytoskeleton through adapter actin-binding proteins including talin and α-actinin, suggesting an association between integrin and cytoskeletal functions (Pavalko and Otey, 1994; Sampath et al., 1998). Treatment of neutrophils with chemotactic agents causes polymerisation of cortical globular actin (G-actin) within seconds, which has no evident function. This is followed by a net reduction and redistribution of the filamentous actin (F-actin) over minutes, which correlates with shape change and migration (Howard and Oresajo, 1985; Watts et al., 1991). Such modifications of the cytoskeleton might influence the function of integrins, for instance by modifying the conformation of the associated integrin and so changing its affinity and kinetics for ligand formation, or by causing active clustering of integrins, which might strengthen binding (Newham and Humphries, 1996; Hughes and Pfaff, 1998). In resting neutrophils, it has been shown that β2-integrin exists in association with talin, that this association is disrupted when talin is cleaved by calpain after neutrophil stimulation, and that

subsequently β2-integrin is found to associate with α-actinin (Sampath et al., 1998). The functional consequences of these rearrangements have not been demonstrated. However, treatment of a T-cell line with calpeptin, an inhibitor of calpain, inhibited activation-induced binding through CD11a/CD18 (Stewart et al., 1998). In an earlier study, Kucik et al. (1996) showed that dissociation of F-actin in a B-cell line, using low doses of cytochalasin D, was associated with greater surface mobility and increased binding of CD11a/CD18. Thus, it has been suggested that activation of integrin binding may occur if the integrin is freed from cytoskeletal restraints, while subsequent re-establishment of linkages might stabilise these bonds (Kucik et al., 1996; Sampath et al., 1998; Stewart et al., 1998).

The small, GTP-binding protein RhoA has been implicated as the upstream effector of cytoskeletal regulation of integrin function in neutrophils (for a review see Williams and Solomkin, 1999). The Rho subfamily is part of the Ras superfamily, which regulates a range of functions of the actin cytoskeleton (Ridley, 1995). In cells other than leukocytes, Rho GTP-ase activity is required for the formation of actin stress fibres (Ridley and Hall, 1992) and also for clustering of integrins (Hotchin and Hall, 1995). Laudanna et al. (1996) found that an inhibitor of Rho (C3-transferase) reduced adhesion of electroporated neutrophils to fibrinogen before or after treatment with chemoattractants, and of lymphoid cells through β1-integrin.

Elucidation of the regulation of CD11b/CD18 requires a kinetic assay capable of dissecting the different phases of adhesion mediated through this integrin. We have previously perfused neutrophils over monolayers of platelets or purified P-selectin to establish rolling adhesion, and then examined the responses of the rolling cells to a range of activators, including formyl peptide (fMLP) (Sheikh and Nash, 1996; Rainger et al., 1997). The neutrophils rapidly became immobilised through activation of constitutive CD11b/CD18, and subsequently downregulated these bonds, but remained attached and migrated using newly expressed CD11b/CD18. The newly expressed CD11b/CD18 was only functional when Mg^{2+} was present at approx. 0.5 mM, and slowly lost function if fMLP was removed. Subsequently, we found that if uncontrolled actin polymerisation was induced with jasplakinolide, neutrophils rolled efficiently but could not immobilise after treatment with fMLP (Sheikh et al., 1997). The present study set out to separate the roles played by rearrangement of the actin cytoskeleton in establishment of bonds through CD11b/CD18, subsequent downregulation of these bonds, and formation of new bonds by de novo expressed integrin. We also wished to distinguish if possible between essentially passive 'liberation' of integrins from constraints, and 'active' control of their conformation and binding. We therefore examined the kinetics of neutrophil immobilisation, migration and detachment after removal of stimulus, and tested how they were modified by agents that affect cytoskeletal structure and linkages: jasplakinolide, C3-transferase, Y-27632 (inhibitor of Rho-kinase), cytochalasin D and calpeptin.

MATERIALS AND METHODS

Reagents

Phosphate-buffered saline contained 1 mM Ca^{2+} and 0.5 mM Mg^{2+} (Gibco, Paisley, UK) and 0.15% bovine serum albumin (Sigma Chemicals, Poole, UK) (PBS/BSA). The following reagents were made up and stored under sterile conditions at the temperatures stated: jasplakinolide (Molecular Probes, Leiden, Holland), 1 mM in ethanol (–20°C); N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma Chemicals), 1 mM in ethanol (–20°C); cytochalasin D (Sigma Chemicals), 1 mg/ml in ethanol (–20°C); calpeptin (Calbiochem, Nottingham, UK), 5 mg/ml in 0.02% DMSO in PBS (–20°C); C3-transferase, expressed and purified as described (Self and Hall, 1995), 0.5 mg/ml in a solution of 150 mM NaCl, 15 mM Tris, 5 mM $MgCl_2$ and 0.1 mM DTT (–80°C); Y-27632 rho kinase inhibitor (gift of Yoshitomi Pharmaceutical Industries, Osaka, Japan), 1 mg/ml in PBS (4°C, used within 1 month).

Isolation of neutrophils

Venous blood was collected from healthy volunteers into EDTA (1.5 mg/ml). Neutrophils were isolated using a two-step density gradient of Histopaque 1077 and 1119 (Sigma Chemicals) as described by Buttrum et al. (1993). The neutrophils were washed twice in PBS/BSA, counted using a Coulter counter (Coulter Electronics, Luton, UK) and adjusted to 1×10^6 cells/ml in PBS/BSA.

Platelet coating of microslides

Microslides (Camlab, Cambridge, UK) are glass capillary tubes having an internal rectangular cross section of 0.3×3 mm and a length of 50 mm. The microslides were initially washed in acid and then coated with 3-aminopropyltriethoxysilane (Sigma Chemicals), to enable platelets to bind to their surface (Buttrum et al., 1993). Blood from the same donor as was used for neutrophil isolation, was collected into sodium heparin (5 U/ml) and platelet-rich plasma was harvested after centrifugation at 400 ***g*** for 5 minutes. Platelets were diluted with PBS to 2×10^8/ml and then loaded into microslides. After 45 minutes at room temperature, the platelets had settled, adhered to the surface and formed an essentially confluent monolayer. Unbound platelets were washed out prior to use.

Flow-based adhesion assay

The adhesion assay was similar to that recently reported by Sheikh and Nash (1996). One end of a microslide, containing a platelet monolayer, was attached to a withdrawal syringe pump via silicon tubing. The other end of the microslide was attached to an electronic microvalve (Lee Products, Gerards Cross, UK), which enabled smooth switching between a reservoir of PBS/BSA and a reservoir containing either a suspension of neutrophils or a chosen chemical agent. The microslide was mounted on the stage of a video microscope enclosed in a perspex box maintained at 37°C. The microslide was perfused at a constant wall shear stress of 0.1 Pa (unless stated otherwise) by controlling the rate of syringe withdrawal.

A 4 minute bolus of neutrophil suspension was perfused through each microslide. During the last minute, six video fields were recorded for 10 seconds each. Following a 1 minute washout with PBS/BSA, the number of neutrophils adherent to the platelet monolayer was counted in 3 full microscope fields of known dimensions, and expressed as adherent cells/mm^2/10^6 cells perfused. In general, total adhesion included both rolling adherent cells and stationary cells. Immediately following initial counts, chemical agents were perfused through the microslides, and the behaviour of adherent neutrophils was video recorded at the desired intervals. Typically fMLP (10^{-7} M) was perfused continuously for 10 minutes, which induced rolling neutrophils to immobilise and migrate over the platelet monolayers (Sheikh and Nash, 1996; Rainger et al., 1997). Finally, adherent neutrophils were recounted. All counts and video recordings were made along the centrelines of microslides.

Video analysis

Neutrophils adherent to platelet monolayers were classified as rolling or stationary. The percentage of adherent cells rolling and their

velocities were analysed off-line from video recordings using custom software and digitised images, as described by Buttrum et al. (1993). These rolling adherent cells typically had velocities of approximately 4 μm/second. Nonadherent cells were visible as streaks on video playback, having velocities of approximately 500 μm/second. Although classified as stationary, neutrophils that were not rolling, i.e. had a velocity of less than 0.4 μm/second, actually migrated slowly over the platelet surface (Rainger et al., 1997). The rate of neutrophil migration was analysed as described by Rainger et al. (1997). In stop frame, the outlines of the initial positions of between 10-14 cells were traced onto transparent acetate sheets placed over a video monitor screen. This tracing was then repeated each time the videotape was advanced 1 minute, typically over a 10 minute period. The size of the monitor screen was calibrated using a stage micrometer, enabling the migration velocities of neutrophils to be expressed in μm/minute.

Neutrophil treatments

Two types of treatment were used: treatment of neutrophils before perfusion, and superfusion of reagents over cells after they had adhered to the platelets. Control samples for all reagents were treated with appropriate concentrations of carriers, ethanol or DMSO.

Neutrophils were incubated with the following reagents before perfusion: jasplakinolide (10 μM, 10 minutes at room temperature; Sheikh et al., 1997); calpeptin (5-300 μM, 30 minutes at 37°C, Stewart et al., 1998); Y-27632 (1-10 μM, 60 minutes at 37°C, Niggli, 1999). To load the poorly permeable C3-transferase we used incubations of up to 6 hours at 37°C or osmotic shock. In the latter case, neutrophils were incubated with C3-transferase (5-20 μg/ml) for 10 minutes at room temperature. Six volumes of sterile, culture-tested water (Baxter, Thetford, UK) were added, followed 30 seconds later by two volumes of 4-times concentrated PBS/BSA. The neutrophils were washed and resuspended at 1×10^6 cells/ml in PBS/BSA. Successful loading of large molecules by this method was demonstrated using FITC-conjugated albumin and flow cytometry (data not shown), or confocal microscopy (where a cytoplasmic but non-nuclear distribution was visible).

After initial adhesion, neutrophils were superfused with fMLP for 10 minutes. In some experiments, after 2 minutes of fMLP there was an 8 minute superfusion of fMLP combined with 10 μM jasplakinolide (in these experiments flow was reduced to a wall shear stress of 0.05 Pa to conserve reagents). After these treatments, adherent neutrophils were superfused with PBS/BSA without fMLP, either with or without Ca^{2+} or Mg^{2+} for a further 10 minutes, in order to study detachment. In other experiments adherent neutrophils were superfused with 1, 5 or 10 mM $MnCl_2$ (Sigma Chemicals) in Hepes-buffered saline (pH 7.35). Hepes buffer was used because Mn^{2+} ions form a precipitate with phosphate ions in PBS. Changing from PBS/BSA to Hepes buffer did not alter the rolling adhesion of unstimulated or jasplakinolide-treated neutrophils (not shown). Alternatively, cytochalasin D (0.1, 0.3, 1 or 10 μM) was superfused over adherent neutrophils for 10 minutes.

Surface expression of CD11b and intracellular levels of F-actin and Ca^{2+}

Expression of CD11b on the surface of neutrophils was quantified by direct immunofluorescence using a phycoerythrin-conjugated antibody (R841; Dako Ltd., Ely, UK) and a FACs scan flow cytometer (model 440; Becton Dickinson Ltd, Cowley, Oxford, UK) as described previously (Sheikh and Nash, 1996). Neutrophils were treated with desired agents and then incubated at 37°C with or without 10^{-7} M fMLP for up to 20 minutes. Samples were then placed on ice and incubated with anti-CD11b for 60 minutes. Samples were washed and then stored in 1% paraformaldehyde. Measurements are expressed as the ratio of median fluorescence intensities (i.e. relative CD11b expression) for treated neutrophils compared to appropriate untreated control cells. F-actin content was quantified for similarly treated neutrophils. Instead of incubation with anti-CD11b, neutrophils were stained with NBD-phallacidin (Molecular Probes, Eugene, OR) using the one-step method of Howard and Meyer (1984). The same flow cytometer was used to measure median fluoresence intensity for treated cells relative to untreated controls.

To detect changes in intracellular Ca^{2+}, neutrophils (1×10^7 cells/ml) were treated with 1 μM FURA2-AM (Sigma) for 15 minutes at 37°C. Cells were washed and incubated with 0 or 10 μM jasplakinolide for 10 minutes, adjusted to 2×10^6 cells/ml with PBS/BSA and placed into a microfluorimeter (Cairn Research, Faversham, UK). Levels of intracellular Ca^{2+} (ratio of fluorescence intensities measured at excitation wavelengths of 340 andv 380 nm) were recorded before and during 5 minutes exposure to 10^{-7} M fMLP.

Statistics

Analysis of variance (ANOVA) was used to assess the dose-dependent effects of treatments. Repeated measures ANOVA was used to assess the effects of time and treatment. Comparisons of individual treatments with controls were made using paired *t*-tests where appropriate. Statistical tests were performed using Statview software (SAS Institute, Cary, USA) and all data are expressed as means ± s.e.m., unless stated otherwise.

RESULTS

Responses of untreated neutrophils

When untreated neutrophils were perfused over platelet monolayers, they adhered efficiently (649±34 cells/mm^2/10^6 perfused cells, 24 experiments). Of the adherent neutrophils, 95±1% were rolling continuously with a velocity of 4.4±0.3 μm/second. Perfusion of fMLP caused approximately 99% of these neutrophils to stop rolling within seconds, as previously reported by Sheikh and Nash (1996). Shortly after this, neutrophils were seen to change shape, spread and migrate over the surface of the platelet monolayers. During continuous perfusion of fMLP, neutrophil migration accelerated up to a

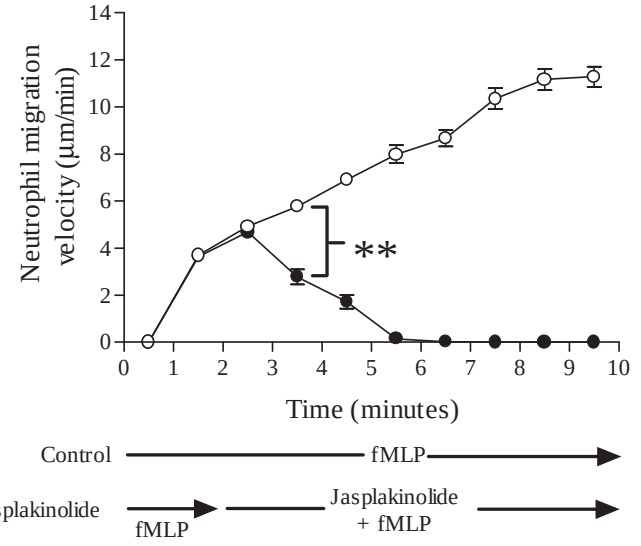

Fig. 1. Effect of jasplakinolide on velocity of migrating neutrophils. Values are means ± s.e.m. of 6 experiments in which neutrophils were perfused with fMLP alone (○) or with fMLP for 2 minutes and then fMLP + jasplakinolide (●). Repeated measures ANOVA showed a significant effect of jasplakinolide ($P<0.001$). After a 1 minute exposure to jasplakinolide, migration velocity was significantly reduced; $^{**}P<0.01$ by paired *t*-test.

Table 1. Effects of jasplakinolide on detachment of neutrophils following 10 minutes continuous superfusion of fMLP and 10 minutes washout

Treatment	Adherent neutrophils (% of value before fMLP treatment)		
	After fMLP	After washout	After Ca^{2+}/Mg^{2+}-free washout
fMLP alone	97.9±2.9	69.2±7.3	47.4±3.7
fMLP + jasplakinolide	99.9±2.8	98.8±2.6*	96.7±3.8**

Neutrophils were perfused over platelets to establish rolling adhesion, and then adherent cells were superfused with fMLP for 10 minutes, or fMLP alone for 2 minutes followed by fMLP plus 10 μM jasplakinolide for 8 minutes. These treatments were followed by washout for 10 minutes with PBS/BSA, or with PBS/BSA lacking Ca^{2+} and Mg^{2+}.

Values are means ± s.e.m., expressed as a percentage of the initial number of adherent cells before perfusion of fMLP (n=6). *P<0.05, **P<0.01; values were significantly different from those obtained with fMLP alone (paired t-test).

maximum velocity of 12.4±0.8 μm/second (Fig. 1) as previously described (Rainger et al., 1997). If fMLP was washed out, after 10 minutes approx. 30% of adherent neutrophils detached in the presence of Ca^{2+} and Mg^{2+}, and approx. 50% detached in the absence of these cations (Table 1). Previous studies by ourselves and others have shown that the initial rolling on platelets is mediated by P-selectin, while immobilisation and migration are supported by CD11b/CD18 (Sheikh and Nash, 1996; Diacovo et al., 1996).

Jasplakinolide blocks integrin activation but 'locks' bound integrins in place

Jasplakinolide is a cell-permeable reagent that induces uncontrolled polymerisation of actin within neutrophils (Sheikh et al., 1997). Pretreatment of neutrophils with 10 μM jasplakinolide did not affect the total number of cells adherent to the platelet monolayers (649±86 cells/mm^2/10^6 perfused cells), the percentage of adherent cells rolling (95±2%) or their rolling velocity (3.8±0.8 μm/second). As shown previously (Sheikh et al., 1997), jasplakinolide pretreatment impaired the response of neutrophils to fMLP, with only 24±6% of cells becoming stationary after 4 minutes (P<0.01, paired t-test, compared to control). The majority of adherent neutrophils continued to roll without any obvious change in shape.

To observe the effect of jasplakinolide on neutrophils that were already migrating, jasplakinolide was combined with fMLP and perfused over neutrophils that had already been exposed to fMLP alone. Perfusion of jasplakinolide rapidly inhibited neutrophil migration. Within 1 minute the average migration velocity of neutrophils exposed to jasplakinolide was half that of the control group, and over 3-4 minutes all migration stopped (Fig. 1). In addition, jasplakinolide inhibited any further shape change of neutrophils, but did not cause them to recommence rolling. During the 10 minute perfusions of fMLP or fMLP combined with jasplakinolide, the number of neutrophils adhered to platelet monolayers did not decrease (Table 1). While washout of fMLP caused detachment of neutrophils that had not been exposed to jasplakinolide, exposure to jasplakinolide almost completely inhibited this

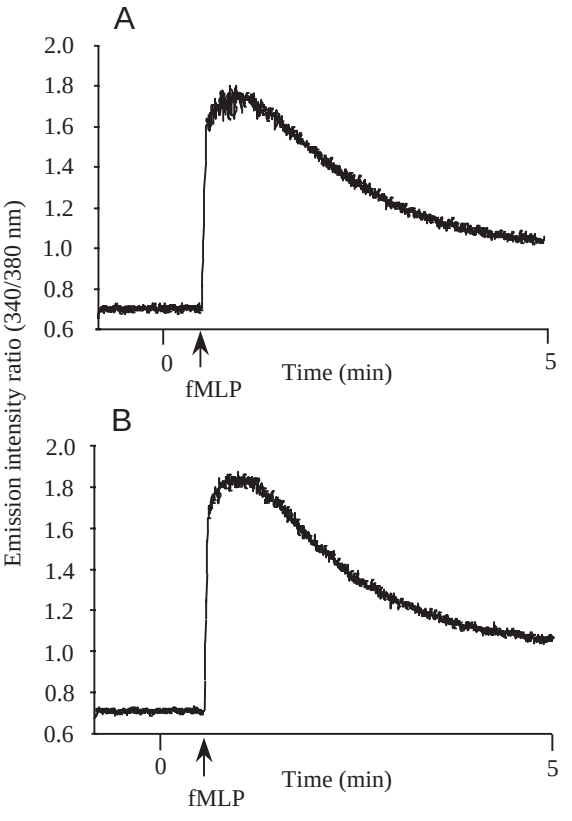

Fig. 2. Effect of jasplakinolide on intracellular Ca^{2+} concentration. Ratios of intensity of emission at two excitatory wavelengths (340/380 nm) are shown before and after addition of 10^{-7} M fMLP for (A) control and (B) jasplakinolide-treated neutrophils. Results shown are typical of experiments performed on two occasions. For details, see Materials and Methods.

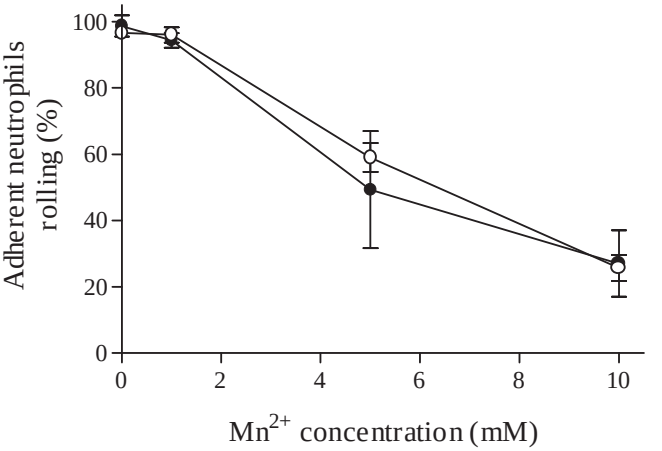

Fig. 3. Effect of jasplakinolide on the conversion of rolling to stationary adhesion of neutrophils induced by Mn^{2+}. Control (○) or jasplakinolide pretreated (●) neutrophils were superfused with increasing concentrations of Mn^{2+}. Values are means ± s.e.m. of 3 experiments.

reversal of attachment, in either the presence or absence of Ca^{2+} and Mg^{2+} (Table 1).

These results show that uncontrolled actin polymerisation has different effects on neutrophil adhesion, depending upon the state of integrin binding at the time of polymerisation. If actin polymerisation takes place before neutrophil stimulation, integrin activation and conversion of rolling to stationary adhesion mediated by CD11b/CD18 is blocked. If, however, CD11b/CD18-ligand binding has already occurred, then polymerisation rapidly inhibits overt signs of further neutrophil activation, and prevents release of CD11b/CD18 upon removal of stimulus, thus 'locking' the neutrophil onto its adhesive surface.

Jasplakinolide does not stop signal transduction or direct external activation of integrins

Uncontrolled actin polymerisation by jasplakinolide might alter neutrophil function by blocking signal transduction or by disrupting integrin structure so that it cannot become activated. To investigate these possibilities, we carried out spectrofluorimetric analysis of the internal Ca^{2+} concentration of neutrophils. Control and jasplakinolide pretreated neutrophils had similar intracellular Ca^{2+} concentrations when quiescent (Fig. 2). Furthermore, upon addition of fMLP, the internal Ca^{2+} concentration increased in parallel for the two groups of cells (Fig. 2). Thus jasplakinolide did not eliminate the intracellular signal transduction mechanism linked to the fMLP receptor. Next, experiments were performed whereby CD11b/CD18 was directly activated by Mn^{2+} (Bohnsack and Zhou, 1992). Untreated or jasplakinolide-treated neutrophils were perfused over platelets to establish rolling adhesion, and then increasing concentrations of Mn^{2+} were superfused over them. The neutrophils converted to stationary adhesion with increasing concentrations of Mn^{2+} and the dose-responses were nearly identical with or without jasplakinolide treatment (Fig. 3). Unlike fMLP-induced stationary adhesion, no shape change or cell migration occurred in either group after perfusion of Mn^{2+}. If Mn^{2+} was washed out, cells resumed rolling and the number adherent did not reduce in either group (data not shown).

It therefore appears that (1) uncontrolled actin polymerisation did not hinder signal transduction by the fMLP receptor, at least up to elevation of the second messenger Ca^{2+}; (2) the external domains of constitutively expressed CD11b/CD18 molecules are still functional in jasplakinolide pre-treated neutrophils, in the sense that they can adopt an activated conformation if acted on directly by an external agent. This implies that cytoskeletal rearrangements may be directly involved in the process of switching CD11b/CD18 to and from its active state.

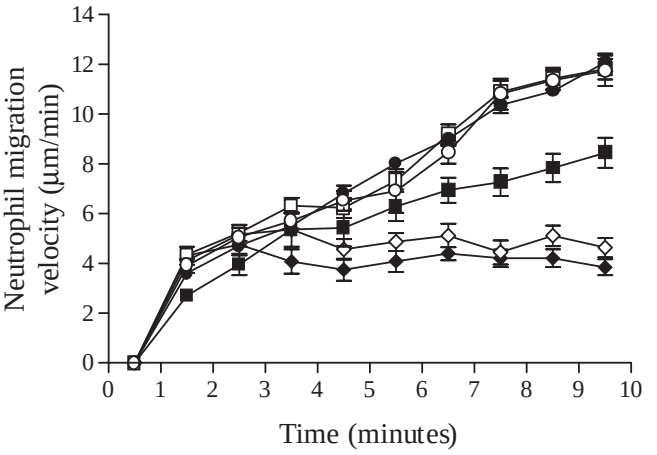

Fig. 4. Effect of C3-transferase on the velocity of neutrophil migration. Neutrophils were untreated (○), exposed to osmotic shock alone (●), or loaded with C3-transferase at 5 μg/ml (□), 7.5 μg/ml (■), 10 μg/ml (◇) or 20 μg/ml (◆). Values are means ± s.e.m. of 3 experiments. Repeated measures ANOVA showed significant effects of time and treatment on migration velocity (P<0.001).

Inhibition of Rho inhibits migration but not integrin-mediated adhesion

Rho-A has been implicated in activation of β_2-integrins (Laudanna et al., 1997). We carried out initial experiments by incubating the inhibitor of Rho, C3-transferase, with neutrophils for up to 6 hours at 37°C. This resulted in a reduced rolling adhesion on platelet monolayers but no change in immobilisation by fMLP (data not shown), although control samples also showed some impairment of initial attachment compared to neutrophils tested immediately after preparation. Osmotic shock loading was therefore used to reduce the incubation period. Compared to the 'shock' control, pretreatment of neutrophils with C3-transferase significantly reduced initial rolling adhesion on platelets in a dose-dependent manner (Table 2). C3-transferase did not, however, affect the response of rolling adherent neutrophils to fMLP, in

Table 2. Effect of C3-transferase on the number of neutrophils adherent before and after exposure to fMLP for 10 minutes, and after washout with PBS/BSA (Ca^{2+}/Mg^{2+}-free)

	Adherent neutrophils (cells/mm^2/10^6 cells perfused)		
Neutrophil pretreatment	Before fMLP	After fMLP	After Ca^{2+}/Mg^{2+}-free washout
None	595±21	576±36	ND
Osmotic shock alone	588±13	582±16	263±38
C3-transferase			
5 μg/ml	577±39	576±41	ND
7.5 μg/ml	416±32**	406±34**	245±19
10 μg/ml	294±35**	285±25**	134±17**
20 μg/ml	301±17**	294±15**	ND

Neutrophils were perfused over platelets to establish rolling adhesion, and then adherent cells were superfused with fMLP for 10 minutes, followed by washout for 10 minutes with PBS/BSA lacking Ca^{2+} and Mg^{2+}.

Values are means ± s.e.m. of 3 experiments. **P<0.01, significantly different from 'shock alone' values (paired t-test).

ND, not done.

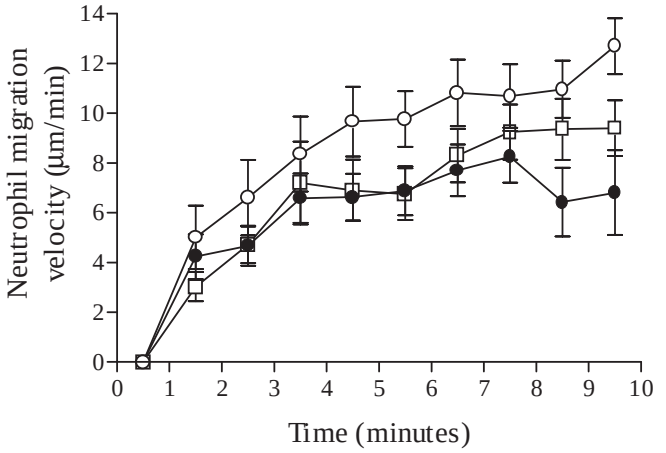

Fig. 5. Effect of rho-kinase inhibitor Y-27632 on velocity of neutrophil migration. Neutrophils were untreated (○), or treated with Y-27632 at 1 μM (●) or 10 μM (□). Values are means ± s.e.m. of 3 experiments. ANOVA showed that treatment with 1 μM significantly affected migration velocity (P<0.01) but that effect of 10 μM was not significant (P=0.11).

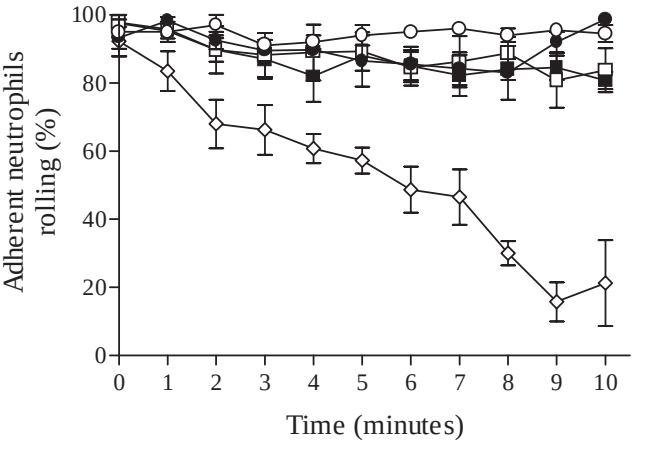

Fig. 6. Variation in the percentage of adherent neutrophils rolling with time during perfusion with cytochalasin D at 0 μM (○), 10^{-7} M (●), 0.3 μM (□), 1.0 μM (■) and 10.0 μM (◇). Values are means ± s.e.m. of 3 experiments. Repeated measures ANOVA showed a significant difference between values at 0 and 10.0 μM cytochalasin D (P<0.01).

the sense that approximately 98% of cells became stationary and underwent a shape change. Neutrophil adherence remained stable throughout fMLP perfusion regardless of treatment with C3-transferase (Table 2). When fMLP was washed out with Ca^{2+}- and Mg^{2+}-free PBS/BSA, approximately half of the adherent neutrophils fell off, with no consistent modification by treatment with C3-transferase (Table 2).

The migration rate of neutrophils was reduced by C3-transferase (Fig. 4). Although the neutrophils were able to migrate slowly, their ability to accelerate was lost in a dose-dependent manner. Migration rate might depend on the rate of actin polymerisation or CD11b upregulation; however, C3-transferase (10 μg/ml) did not impair the increase in F-actin content or CD11b expression induced by fMLP. In control neutrophils, F-actin (assessed using NBD-phallacidin) was increased by 91% above baseline after treatment with fMLP for 2 minutes, and this increase fell back to 48% above baseline after treatment for 10 minutes (means of three experiments). The increases were 130% and 97% for neutrophils loaded with C3-transferase in paired experiments. Expression of CD11b was increased by 120% for control neutrophils exposed to fMLP for 10 minutes, while the increase was 110% for neutrophils that had been pretreated with C3-transferase (means from 3 experiments).

The technique of osmotically shocking neutrophils itself had no affect on initial rolling adhesion or migration compared to untreated neutrophils kept isotonic (Table 2, Fig. 4). Thus, C3-transferase impairs selectin-mediated capture, but not integrin-mediated immobilisation induced by fMLP. Treated neutrophils were able to migrate only slowly, but were not 'locked' in place since they detached upon washout of fMLP.

To test effects downstream of Rho, neutrophils were pretreated with the rho kinase inhibitor Y-27632. This had no significant affect on the number of cells rolling adherent on platelet monolayers, either before or after 10 minutes perfusion with fMLP, compared to untreated control (data not shown). Y-27632 also had no effect on conversion to stationary adhesion or shape change in response to fMLP. It did, however, cause a slight reduction in neutrophil migration velocity (Fig. 5).

Cytoskeletal disruption alone fails to induce integrin-mediated adhesion

It has been suggested that adhesion through CD11a/CD18 can be promoted through dissociation of links to the actin cytoskeleton (Kucik et al., 1996; Stewart et al., 1998). To investigate whether this mechanism could cause rapid immobilisation of neutrophils, cytochalasin D was perfused over rolling adherent neutrophils, but little effect on the percentage of adherent neutrophils rolling was seen for doses of cytochalasin D up to 1 μM (Fig. 6). This was the concentration range previously found to promote attachment of

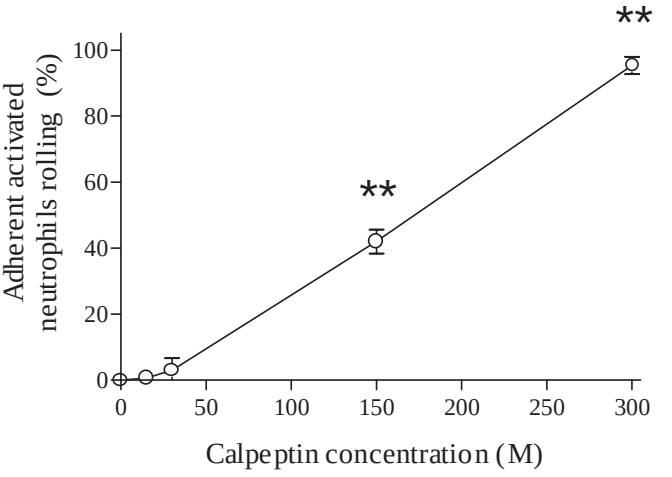

Fig. 7. Effect of calpeptin on the conversion of neutrophil rolling to stationary adhesion by fMLP. fMLP was perfused over cells and the percentage remaining rolling was analysed after 4 minutes. Values are means ± s.e.m. of 3 experiments. ANOVA showed a significant effect of treatment; **P<0.01 for 150 μM or 300 μM calpeptin, compared to the untreated control.

lymphoid cells in a stationary adhesion assay (Kucik et al., 1996). At a higher concentration of 10 μM cytochalain D, neutrophils assumed a teardrop shape aligned in the direction of flow, and converted to stationary adhesion within minutes (Fig. 6). This response is similar to that reported previously, but shown not to be mediated by β2-integrins (Sheikh and Nash, 1998). We checked that the lower concentrations were active in our assay by first perfusing fMLP over rolling neutrophils for 5 minutes to induce migration, and then switching to fMLP mixed with cytochalasin D (0.3 or 1.0 μM). Migration gradually slowed over the next 5 minutes compared to controls continuously perfused with fMLP. This agrees with previous reports that approx. 1 μM cytochalasin D largely inhibits actin polymerisation in neutrophils responding to fMLP (Howard and Oresajo, 1985).

Calpain has been identified as an activation-dependent modifier of links between β2-integrins and the cytoskeleton (Sampath et al., 1998; Stewart et al., 1998). Treatment of neutrophils with calpeptin, a potent inhibitor of calpain, had no effect on initial adhesion to platelets, the percentage of adherent cells rolling and the rolling velocity, compared to untreated controls (data not shown). Conversion to stationary adhesion in response to fMLP was significantly inhibited by calpeptin at 150-300 μM (Fig. 7). However, it was notable that the treated neutrophils were unable to change shape in response to fMLP. When we measured F-actin content of neutrophils, it was found that calpeptin treatment abolished the increase induced by fMLP (two separate experiments; data not shown). The active concentrations of calpeptin were several orders of magnitude higher than the ID_{50} for calpain (supplier's literature). We checked whether migration was modified for the lower concentrations (approx. 10 μM), which allowed immobilisation in response to fMLP. Migration rate was not affected (data not shown).

DISCUSSION

Regulation of the binding of β2-integrins to their ligands is important during leukocyte adhesion to the vessel wall and migration into tissue. Both CD11a/CD18 and CD11b/CD18 are involved in adhesion and migration of neutrophils on endothelial cells (Smith et al., 1989), while CD11b/CD18 alone mediates immobilisation of activated neutrophils on platelets and proteins such as albumin and fibrinogen (Altieri, 1991; Hughes et al., 1992; Sheikh and Nash, 1996). Links to the actin cytoskeleton modulate integrin function in a number of more sedentary cells, for instance through formation of focal adhesion complexes (see e.g. Hemmings et al., 1995, for a review). Integrins are also linked to the cytoskeleton in leukocytes, and an intact cytoskeleton is required for clustering of these receptors (Haverstick et al., 1992; Pavalko and Otey, 1994; Peter and O'Toole, 1995; Sampath et al., 1998). There is little direct evidence, however, that changes in actin structures regulate rapid responses of neutrophil integrins, and CD11b/CD18 in particular. Here, we have demonstrated that rapid upregulation and subsequent downregulation of the adhesive function of CD11b/CD18 in neutrophils is effected through the actin cytoskeleton. The process appears to be distinct from changes in adhesion that are attributable to clustering or movement of receptors in the membrane plane, and from cytoskeletal rearrangements regulated through RhoA, which influenced the rate of migration but not adhesion itself.

Uncontrolled polymerisation of actin by jasplakinolide blocked rapid activation of CD11b/CD18-mediated adhesion in neutrophils stimulated with fMLP, and also disallowed detachment of CD11b/CD18 once ligand binding had already occurred. The effect was not due to a failure of signal transduction, judged by the generation of intracellular Ca^{2+} transients. In our hands, chelation of intracellular Ca^{2+} with a high concentration of cell-permeant BAPTA-AM ablates immobilisation of rolling neutrophils in response to fMLP (G. B. Nash, unpublished observations). Retention of the Ca^{2+} response is thus an important test for an agent postulated to modify integrin activation through cytoskeletal linkages. In addition, integrin-mediated binding was still inducible by Mn^{2+}, so that jasplakinolide had not caused a structural rearrangement of the integrin that disallowed binding. Since jasplakinolide inhibits de novo surface presentation of CD11b/CD18 (Sheikh et al., 1997), the results indicate that the function of constitutive CD11b is modulated by active rearrangement of the cytoskeleton.

We did not find evidence that CD11b/CD18 was rapidly activated by a 'passive' response, in which disruption of integrin anchorage to the cytoskeleton increases mobility in the membrane and increases the probability of binding ligand. Rolling neutrophils did not form integrin-mediated bonds when treated with cytochalasin D. In static systems, cytochalasin D has been found to increase levels of adhesion mediated by CD11a/CD18 or CD11b/CD18 in unstimulated leukocytes (Elemer and Edginton, 1994; Kucik et al., 1996) and to decrease it in cells exposed to activators (Haverstick et al., 1992; Peter and O'Toole, 1995). In the former case, freed integrins may have been able to form more low-affinity bonds and stabilise adhesion, while in the latter, active capping of the receptor was disabled (Peter and O'Toole, 1995). Rapid transformation of binding characteristics of individual CD11b/CD18 receptors does not, however, appear to be affected by cytochalasin treatment. The calpain inhibitor calpeptin did impair functional activation of CD11b/CD18, in agreement with a previous report on the function of CD11a/CD18 (Stewart et al., 1998). Calpain has been suggested to promote activation of β2-integrins by freeing them from immobilisation through talin (Stewart et al., 1998; Sampath et al., 1998). We found, however, that calpeptin-treated neutrophils did not change shape or increase their content of F-actin when exposed to fMLP. Thus, rather than inhibiting mobilisation, calpeptin may have inhibited conformational changes in CD11b/CD18 through a wider effect on the cytoskeleton. In fact, conformational change and mobilisation are not mutually exclusive, and might play separate roles in rapid changes and more gradual modifications of adhesion.

Perhaps unexpectedly, inhibition of RhoA by C3-transferase did not block the rapid immobilisation or longer-term stability of adhesion of activated neutrophils. It was previously reported that C3-transferase reduced the binding of electroporated neutrophils to fibrinogen (presumably mediated by CD11b/CD18), both with and without treatment with fMLP in a static system (Laudanna et al., 1996). It is possible that RhoA was involved in cytoskeletal-driven integrin clustering, which stabilised adhesion in that model, and/or that electroporation

itself modified the mechanisms by which neutrophil adhesion was regulated. We did find that treatment with C3-transferase impaired selectin-mediated attachment of neutrophils to platelets and markedly reduced rate of migration after treatment with fMLP. The loss of motility is consistent with an earlier study of electroporated bovine neutrophils (Stasia et al., 1991). Rate of migration will depend on the kinetics of integrin up- and downregulation and of cycles of actin polymerisation-depolymerisation (Huttenlocher et al., 1996). Here, treatment with C3-transferase did not appear to affect the rate of 'turnover' of integrins; it failed to modify the increase in surface expression of CD11b induced by fMLP, or the rate of detachment of neutrophil integrins when fMLP was removed. Since the fMLP-induced increase in intracellular F-actin was not inhibited by C3-transferase, the likely route by which RhoA affected migration rate was through control of cycling between F- and G-actin.

The inhibitor of Rho-kinase (Y-27632) was less effective than C3-transferase in decreasing migration rate. The implication is that the downstream, functional effects of Rho operated only partially through Rho kinase. Niggli (1999) showed marked inhibition of migration by Y-27632, but using an assay very different from the one used here. Neutrophils were activated with low concentrations of fMLP (10^{-9}M), the fMLP was washed out, and migration was observed after sedimentation onto a protein-coated surface. The driving signal for migration was thus much lower than in our model (where 10^{-7}M fMLP remained present) and the migration was much slower. The concentrations of Y-27632 used here (1-10 μM) spanned the active range previously described (Niggli, 1999).

The above results have implications for the mechanisms of by which integrin binding is up- and downregulated. Functional 'activation' of β2-integrins might occur through conformational change in the receptor opening a binding site, enzymatic modification of the receptor or an associated protein, increased mobility of receptor in the membrane and movement of receptors into clusters (see Newham and Humphries, 1996; Hughes and Pfaff, 1998, for reviews). A mechanism that could stabilise binding in a stationary cell would not necessarily allow rapid transformation from rolling to stationary adhesion. Integrins have relatively slow forward and reverse rate constants (Masson-Gadais et al., 1999) compared to selectins (e.g. Alon et al., 1995). Even when activated, they do not allow capture of fast-flowing neutrophils (Lawrence et al., 1990; Perry et al., 1993), but can immobilise cells that are already moving slowly. The implication is that inactive β2-integrins on circulating neutrophils have a low affinity (i.e. the ratio of forward/reverse rate constants), and that upon activation affinity suddenly increases. The forward rate constant is still only adequate for efficient binding of relatively slow-moving cells. It is unlikely that mobilisation of integrin in the cell surface or active clustering could induce the rapid transformation of binding. While the likelihood of meeting ligand might increase, the integrins on rolling cells are effectively 'sweeping' the surface rapidly on a molecular scale in any case. Recent studies suggest that such convective processes are more effective in bringing receptors together than diffusion on the surface (Chang and Hammer, 1999). Clustering may increase 'avidity' or stabilise adhesion by effectively sharing disrupting forces between closely packed bonds, making it more difficult for cells to 'peel' from a surface (e.g. Ward et al., 1994), but it is unlikely to cause immobilisation itself, however, unless intrinsic integrin binding characteristics have been transformed. Clearly, clustering and increased mobility are not actually required for functional activation, judging from the ability of external Mn^{2+} to induce it. Thus, a change in integrin conformation can better explain the dramatic transition from rolling to stationary adhesion. Subsequent loss of integrin bonds to allow migration, may arise from the intrinsic lifetime of the bonds, reversal to a low affinity conformation, or forced breakage of bonds or extraction of integrin from the membrane. Our finding that jasplakinolide inhibited reversal of adhesion suggests that this phase can also be actively regulated.

It is tempting to suggest that the polymerisation of cortical actin which rapidly follows neutrophil stimulation (Howard and Oresajo, 1985) might shift integrin conformation through linkage proteins. Previous studies showed that β2-integrin is linked to talin in resting neutrophils, and that within minutes of cell activation this link is lost, and CD18 is found to be linked to α-actinin (Sampath et al., 1998). It was proposed that the freed integrin could more efficiently bind ligand and that the bonds could become stabilised by the new linkage. However, it was not demonstrated that the CD18 linked to talin or α-actinin were the same population of receptors. For instance, one might propose that the initial activation of constitutive integrin is driven through talin (allowing immobilisation within seconds), and that downregulation of this existing bond occurs though cleavage (allowing detachment over minutes). Formation of new linkages to α-actinin may be essential for function of newly expressed CD11b/CD18 (allowing prolonged attachment and migration). This hypothesis is amenable to experimental test if one can specifically modify these links and test their roles in the different stages of neutrophil immobilisation and migration. In conclusion, control of integrin-mediated adhesion and actin polymerisation must be co-ordinated in leukocytes to allow efficient migration, and our results suggest that they are directly linked.

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