Engagement of L-selectin impairs the actin polymerizing capacity of β2-integrins on neutrophils

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SUMMARY

A sequential activation of L-selectin and β2-integrins on neutrophils is crucial for the rolling, adherence and subsequent migration of these cells on the endothelium. However, little is known about a possible interplay between these adhesion receptors in the final regulation of cell motility. The results presented here show that sulfatides themselves achieved by a complicated interplay between adhesion receptors in the final regulation of cell motility. The results presented here show that sulfatides themselves (here used as tools to activate L-selectins), have no major effect on the cellular content of filamentous actin (F-actin), but cause a time-related decrease in the β2-integrin-induced formation of F-actin. This effect of sulfatides was abolished in cells lacking L-selectin as a result of pretreatment with chymotrypsin. A similar sulfatide-induced activation of L-selectin also caused a pronounced and time-related decrease of a subsequent chemotactic peptide-induced F-actin response. The effect of sulfatides on both β2-integrin- and chemotactic peptide-induced F-actin were abolished if L-selectin were blocked by preincubating the cells with specific antibodies to L-selectin. These effects of L-selectin engagement on cellular F-actin content were neither abolished by blocking the cytosolic free Ca2+ signal with bis-(2-amino-5-methylphenoxy)ethanone-N,N,N′,N′-tetraacetic acid tetraacetoxymethyly ester (MAPT/AM) nor by blocking a cAMP-induced activation of protein kinase A by pretreating the cells with adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS). Instead we found that L-selectin engagement impaired an early β2-integrin-induced tyrosine kinase activation, an event shown to be necessary for a normal β2-integrin-mediated F-actin response. The present demonstration of a negative feed-back function of L-selectin on β2-integrin-induced modulations of the actin cytoskeleton, suggests that the relative distribution and/or density of the respective L-selectin and β2-integrin ligands on endothelial cells might be important factors in determining the final site of firm adhesion and extravasation of neutrophils.

Key words: Neutrophil, β2-integrin, L-selectin, Actin polymerization

INTRODUCTION

Recruitment of neutrophils into extracellular tissues is mediated by highly specific interactions between circulating neutrophils and the vascular endothelium at sites of extravasation. This process of extravasation can be divided into at least three distinct phases (Butcher, 1991; Lasky and Rosen, 1992). The initial phase is the transient adhesion ‘rolling’ of neutrophils along the endothelium. This rolling process is mediated by the L-selectin on neutrophils and its counter-ligands that are either constitutively or rapidly induced on the endothelium at inflammatory foci. This behaviour causes neutrophils to slow down and thereby increases the possibility of establishing an integrin-mediated firm adhesion to the vessel wall (von Andrian et al., 1991; Lawrence and Springer, 1991). Ultimately, neutrophils will, under the influence of chemotactic factors, extravasate across the vessel wall and continue to migrate along chemotactic gradients toward an inflammatory focus. This scenario of neutrophil diapedesis is achieved by a complicated interplay between adhesion molecules and chemoattractant receptors. Indeed, there have been reports indicating the existence of cross-talk between chemoattractant and adhesion receptors, whereby chemotactic factors can modulate both the adhesive properties of neutrophils (Luscinskas et al., 1992) and the signal transduction capacity of the β2-integrins on these cells (Eierman et al., 1994).

The first report on the intracellular signalling capacity of L-selectin demonstrated that this molecule is capable of inducing an intracellular Ca2+ signal that is responsible for an enhanced expression of tumor necrosis factor-α (TNFα) and interleukin-8 mRNA in human neutrophils (Laudanna et al., 1994). It was later shown that specific antibody cross-linking of L-selectin on human neutrophils enhanced a subsequent oxidative burst induced by N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMet-Leu-Phe) or TNFα (Waddell et al., 1994). Despite these intriguing observations, no attempt has yet been made to elucidate the potential role of L-selectin-induced signalling in the regulation of the locomotory response of neutrophils.

The motile properties of neutrophils are primarily deter-
mained by a continuous remodeling of the microfilamentous system (Stossel, 1993). The ability of chemotactic factors to induce a rapid and transient increase in cellular F-actin content of neutrophils has been recognized (Wallace et al., 1984; Omann et al., 1987). It has also been shown that both neutrophil adherence (Southwick et al., 1989) and specific antibody engagement of $\beta_2$-integrins on non-adherent neutrophils (Löfgren et al., 1993) caused a relatively sustained F-actin response. In a number of different cell types, including neutrophils, such integrin engagement has been found to result in increased tyrosine kinase activity (Kornberg and Juliano, 1992; Berton et al., 1994; Hellberg et al., 1995). Of particular interest for the present study was the finding of a link between tyrosine kinase activation and cytoskeletal rearrangements (Chrzanowska-Wodnicka and Burridge, 1994; Ridley and Hall, 1994; Barry and Critchley, 1994) as well as integrin-directed cell motility (Klemke et al., 1994).

The sustained effect of $\beta_2$-integrin engagement on F-actin content is necessary for firm adhesion of neutrophils but this effect on migration is unclear. Nonetheless, the chemotactic peptide receptor but not $\beta_2$-integrins has been found to trigger a cAMP signal and addition of cAMP to electroporameised neutrophils could reverse the $\beta_2$-integrin-induced sustained increase in cellular F-actin content (Löfgren et al., 1993). These findings suggest that the modulation of the actin network in neutrophils occurs as a result of a delicate second messenger cross-talk between $\beta_2$-integrins and chemotactic-factor receptors. This idea can be extended to include L-selectin adhesion molecules, since they are responsible for the initial rolling of neutrophils along the vessel wall; an event that precedes adherence and migration. Although neutrophils apparently retain their round shape while rolling (von Andrian et al., 1991), nothing is known about how L-selectin-dependent rolling might affect $\beta_2$-integrin-mediated modulation of the neutrophil actin network.

Consequently, the specific aim of the present investigation was to study how engagement of the L-selectin adhesion molecule on human neutrophils would affect the ability of $\beta_2$-integrins to modulate the actin network of these cells.

**MATERIALS AND METHODS**

**Materials**

Chemicals were purchased as follows: dextran and Ficoll-Paque, Pharmacia (Sweden); fluorescein-labelled phallolidin, fura2/AM, Molecular Probes Inc. (Oregon, USA); chymotrypsin, f-Met-Leu-Phe, sulfatides, sodium orthovanadate, lysophosphatidylcholine, galactocerebrosides, Sigma Chemical Co. (St Louis, USA); hydrogen peroxide, Janssen Chimica (Belgium); methyl-2,5-dihydroxycinnamate (erastatin analogue), Calbiochem (San Diego, USA); Rp-cAMPS, BioLog (Germany); phorbol 12-myristate 13-acetate (PMA), LC Serv. Corp. (Woburn, USA); gelvatrol, Monsanto (St Louis, USA); mAb TQ1 (anti-CD62L; IgG 1 ; Tedder et al., 1990), Coulter Immunology (Hialeah, USA); rabbit anti-mouse immunoglobulins (RAM), anti-HLA class I antigen (clone W6/32; IgG2a), Dakopatts (Denmark). The mAb IB4 (anti-CD18; IgG2a) originated from Dr S. Wright (Rockefeller University, USA; Wright et al., 1983). The anti-phosphotyrosine mAb (clone 4G10) was from Upstate Biotechnology Inc. (Lake Placid, USA), the peroxidase-conjugated goat anti-mouse IgG was obtained from Jackson Immunoresearch Lab. Inc. (West Grove, USA). The enhanced chemoluminescence kit (ECL) was from Amersham International (UK).

**Isolation of neutrophils**

Neutrophils were prepared from healthy human donors essentially as previously described (Böyum, 1968). Following dextran sedimentation, centrifugation through Ficoll-Hypaque and hypotonic lysis of red cells, neutrophils were washed and resuspended in a medium with the following composition: 136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.1 mM CaCl2, 0.1 mM EGTA, 1.2 mM KH2PO4, 5 mM NaHCO3, 5.5 mM glucose and 20 mM Hepes, pH 7.4. This medium is referred to as Ca2+ medium throughout the paper.

**Activation of adhesion molecules by antibody cross-linking**

Neutrophils (4×106 cells/ml) were incubated with the mAb IB4 (10 μg/ml), directed against CD18, for 20 minutes at 37°C in Ca2+ medium. In addition, the $\beta_2$-integrins were preactivated by the presence of 1 nM PMA in the medium during the last 10 minutes of this incubation since this treatment of PMA had no effect on its own but potentiated the signalling and F-actin responses induced by $\beta_2$-integrins (Fällman et al., 1993; Löfgren et al., 1993). However, the effect of sulfatides on $\beta_2$-integrin-induced F-actin response were also observed in the absence of PMA (data not shown). The cells were then washed and resuspended in prewarmed Ca2+ medium. Cross-linking of the IB4 mAb was achieved by exposing the cells to RAM (1:50 dilution). Control experiments were performed using F(ab')2 fragments of both RAM and the mAb IB4 to exclude the possibility that the observed responses were due to Fc-receptor engagement. The control experiments revealed that addition of F(ab')2 fragments of both the mAb IB4 and RAM caused a Ca2+ transient, a F-actin response and tyrosine phosphorylations (data not shown), which were similar to the data obtained with intact antibodies.

**Determination of the cellular content of F-actin**

The cellular content of F-actin was analysed by staining with fluorescein-phalloidin as previously described (Löfgren et al., 1993). The fluorescence of the samples was determined in a SPEX spectrofluorometer (excitation set at 488 nm and emission at 522 nm).

**Determination of the cytosolic free Ca2+ level**

Neutrophils were loaded with the Ca2+ indicator dye fura2, as previously described (Andersson et al., 1986). In short, cells were incubated with 2 μM of fura2/AM at 37°C for 20 minutes in a Ca2+ medium. In experiments in which the cells were also incubated with antibodies (IB4, anti-CD18 mAb or TQ1, anti-CD62L mAb), fura2/AM and antibodies were present at the same time. Measurements of fluorescence were carried out using a SPEX spectrophuorometer. The cuvette holder was thermostated to 37°C and equipped with a continuous stirring device. Cytosolic free Ca2+ concentrations were calculated on the basis of the fluorescence ratio (1 per second) obtained by rapidly alternating the excitation wavelength between 340 and 380 nm while keeping the emission wavelength at 510 nm (Gryniewicz et al., 1985).

**Coating of coverslips with intercellular adhesion molecule 1**

Coverslips were coated with recombinant intercellular adhesion molecule 1 (ICAM-1) obtained from a membrane extract of S2 cells overexpressing ICAM-1. The ICAM-1 extract was dialyzed and purified by chromatography on a column of anti-ICAM-1. The coverslips were coated by placing them in a Petri dish containing ICAM-1 (40-50 mg/ml) suspended in tris-buffered saline (TBS), pH 7.6, overnight at 4°C under continuous rotation. The coverslips were washed in TBS to remove excess ICAM-1. Any uncoated areas on the coverslips were blocked for an hour with BSA (3%, v/v) in TBS at 4°C. The coverslips were washed and prewarmed to 37°C in Ca2+ medium prior to the addition of neutrophils.
Preparation of cells adhering to an ICAM-1 surface for confocal microscopy

Neutrophils (1×10⁶ cells/ml) were allowed to settle on a coated coverslip for 20 minutes at 37°C; when sulfatides were used, they were added to the cells during the final minute of the incubation period. Unattached cells were washed away, after which the cells were fixed in methanol/acetone (1:1) at −20°C for 10 minutes. Before further use, the fixative was allowed to evaporate at room temperature. The coverslips were then rinsed in PBS, pH 7.6, and placed in a solution of rhodamin-phalloidin (0.6 µg/ml) and incubated in the dark for 30 minutes at room temperature. The stained adherent cells were washed in PBS and then mounted on glass slides in a medium consisting of 80 ml PBS and 40 ml glycerol (87%, w/w) supplemented with 20 g of gelvatrol.

Confocal microscopy

The preparations were viewed in a Zeiss inverted Laser Scan Confocal Microscope, LSM 410, equipped with an argon laser with excitation wavelength of 488 nm. Images were recorded at wavelengths above 590 nm, starting from the top (assigned A for control and E for sulfatide treated cells in Figs 4 and 5) and going to the bottom of each cell.

Gel electrophoresis and western blot analysis

Prior to electrophoresis, all samples were boiled for 5 minutes in SDS-PAGE sample buffer consisting of 125 mM Tris-HCl, pH 6.8, 8% (v/v) glycerol, 2.5% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.05% (w/v) bromphenol blue. Electrophoresis was performed in 7.5% polyacrylamide gels (Laemmli, 1970), and the resolved proteins were electrophoretically transferred to nitrocellulose membranes. For immunological detection, the nitrocellulose membranes were incubated overnight with 5% (w/v) BSA in PBS, pH 7.3, and then incubated for an additional 30 minutes with 5% (w/v) non-fat dried milk and 5% (w/v) BSA in PBS, pH 7.3, to block non-specific protein binding. The amount of tyrosine phosphorylated proteins was detected by using a commercial ECL kit obtained from Amersham.

Preparation of sulfatides and pervanadate

Sulfatides were prepared in a micelle form (5 mg/ml in PBS and sonicated for 3 minutes). It has been established that they are useful as activators (tools) of L-selectins and such a preparation will cause a multivalent cross-linking of L-selectin (Foxall et al., 1992; Laudanna et al., 1994). For control experiments galactocerebrosides were prepared in an identical manner.

Pervanadate was prepared by mixing equimolar concentrations of hydrogen peroxide and sodium orthovanadate for 15 minutes at 22°C. This was followed by the addition of catalase (200 µg/ml) to remove residual hydrogen peroxide (Imbert et al., 1994).

RESULTS

The actin polymerizing capacity of β2-integrins is impaired by sulfatide-induced activation of L-selectin

Exposing non-adherent neutrophils to sulfatides caused if any response, a small decrease in the cellular content of F-actin (Fig. 1A). This effect of L-selectin engagement on the cellular F-actin response is in direct contrast to the well known effect of the chemoattractant, fMet-Leu-Phe, which causes a rapid polymerization of F-actin (Fig. 6A; Wallace et al., 1984) and the sustained F-actin response found upon engagement of activated β2-integrins (Fig. 1A; Löfgren et al., 1993).

To study the sequential interplay between the L-selectin and β2-integrins on neutrophils, the cells were treated with sulfatides for 1 or 10 minutes prior to the engagement of β2-integrins. Treatment of neutrophils with sulfatides for 1 minute caused a marked decrease in the ability of β2-integrins to induce an increase in the cellular content of F-actin (Fig. 1A). The β2-integrin-induced F-actin response was partially restored if the cells were exposed to sulfatides for a longer period. The coverslips were then rinsed in PBS, pH 7.6, and placed in a solution of rhodamin-phalloidin (0.6 µg/ml) and incubated in the dark for 30 minutes at room temperature. The stained adherent cells were washed in PBS and then mounted on glass slides in a medium consisting of 80 ml PBS and 40 ml glycerol (87%, w/w) supplemented with 20 g of gelvatrol.
Effect of chymotrypsin pretreatment on the percentage of the time-zero value for each individual experiment. Methods. Values are given as mean ± s.e.m (indicated times was determined as described in Materials and Methods. Trace 1 shows the Ca²⁺ transient induced when chymotrypsin-pretreated neutrophils were incubated with fura2/AM and PMA as described in Materials and Methods. Cells were pretreated with chymotrypsin as described above, and incubated with the mAb IB4, PMA as described in Materials and Methods. The cells were then exposed to medium alone (□) or sulfatides for 1 minute (■) before the addition of RAM. The cellular F-actin content at the indicated times was determined as described in Materials and Methods. Values are given as mean ± s.e.m (n=6) and expressed as a percentage of the time-zero value for each individual experiment.

To confirm that the effects of sulfatides are mediated through L-selectin was also tested by pretreating the cells with the TQ1 mAb, which recognizes an epitope on the L-selectin molecule (Tedder et al., 1990). Pretreating neutrophils with a saturating concentration of this mAb blocked the sulfatide-induced impairment of the F-actin response induced by β₂-integrin engagement (Fig. 3A). This blocking effect was not seen if the cells were pretreated with an antibody against another cell surface antigen such as HLA class I, prior to treatment with sulfatides (Fig. 3A). The blocking capacity of the mAb TQ1 was also tested on sulfatide-induced cytosolic free Ca²⁺ signalling. If the cells were first pretreated with the mAb TQ1, the ability of sulfatides to induce a cytosolic free Ca²⁺ transient was abolished (Fig. 3B). The described results suggest that the sulfatide-induced effects are mediated via L-selectin, a conclusion previously made by other investigators (Foxall et al., 1992; Laudanna et al., 1994).

An alternative approach to study the interplay between L-selectin and β₂-integrins and the effects of these receptors on the actin network of neutrophils is illustrated in Fig. 4. In the

Fig. 2. (A) Effect of chymotrypsin pretreatment on the sulfatide-induced effect on the β₂-integrin-induced F-actin response. Human neutrophils were incubated with chymotrypsin (100 units/ml) for 5 minutes at 37°C. After washing, the cells were incubated with the mAb IB4 and PMA as described in Materials and Methods. The cells were then exposed to medium alone (■) or sulfatides for 1 minute (□) before the addition of RAM. The cellular F-actin content at the indicated times was determined as described in Materials and Methods. Values are given as mean ± s.e.m (n=6) and expressed as a percentage of the time-zero value for each individual experiment. (B) Effect of chymotrypsin pretreatment on the β₂-integrin-induced cytosolic free Ca²⁺ response. Cells were pretreated with chymotrypsin as described above, and incubated with the mAb IB4, fura2/AM and PMA as earlier described. Fluorescence analysis was carried out as described in Materials and Methods. Traces 1 and 2 show the Ca²⁺ transients induced by β₂-integrin engagement (arrow) in non-pretreated cells (medium alone) and in chymotrypsin-pretreated cells, respectively. (C) Effect of chymotrypsin pretreatment on the sulfatide-induced cytosolic free Ca²⁺ response. Cells were pretreated with chymotrypsin as described above, and incubated with fura2/AM. Fluorescence analysis was carried out as described in Materials and Methods. Trace 1 shows the Ca²⁺ transient induced by exposing non-pretreated cells to sulfatides Trace 2 shows the Ca²⁺ transient induced when chymotrypsin-pretreated cells were exposed to sulfatides. The arrow indicates addition of sulfatides. Traces shown are representative of at least 3 separate experiments.
experiments described in the figure legends, neutrophils were allowed to adhere and move on an ICAM-1 pre-coated surface. ICAM-1 is a natural ligand for $\beta_2$-integrins (Ruoslahti, 1991; Hynes, 1992), and it therefore provides an alternative means of $\beta_2$-integrin-activation. Confocal microscopy of F-actin stained cells moving on an ICAM-1 precoated surface revealed that the majority of attached cells exhibited a polarized appearance with an extensive network of F-actin predominantly in the leading front of the cells (Fig. 4A-D). If sulfatides were present a major decrease in the cellular F-actin content could be visualized (Fig. 4E-H). The data obtained with the confocal microscopy were also analysed quantitatively by measuring the number of positive pixels within a defined frame of a constant size placed over each single cell. As can be seen in Fig. 5 this
analysis confirms the impression obtained from the representative cells outlined in Fig. 4. In addition, and perhaps more importantly, these data imply that L-selectin engagement can affect the β2-integrin-induced F-actin formation, even when these filaments are continuously formed during cell locomotion.

**The actin polymerizing capacity of chemotactic peptide receptors is also impaired by sulfatide-induced activation of L-selectin**

Treatment of neutrophils with sulfatides for 1 minute markedly decreased the ability of fMet-Leu-Phe to induce an increase in the cellular content of F-actin (Fig. 6A). The fMet-Leu-Phe-induced F-actin response was partially restored (peak level around 144%) when the cells were exposed to sulfatides for a longer period of time (10 minutes; Fig. 6A). The dose-response analysis of the sulfatide-induced effect on the fMet-Leu-Phe-induced F-actin response (Fig. 6B) is similar to that previously described for the β2-integrin-induced F-actin response (Fig. 1B).

We tested the involvement of L-selectin by pretreating the cells with a saturating concentration of the mAb TQ1. As can be seen in Fig. 6C the sulfatide-induced impairment of the F-actin response induced by fMet-Leu-Phe was abolished by such pretreatment. This experiment suggests that the sulfatide-induced effect on the fMet-Leu-Phe-induced F-actin response was also mediated via L-selectin.
Targets for sulfatide-mediated inhibition of β2-integrin-induced actin polymerization

It has previously been shown that an elevated cytosolic free Ca²⁺ level causes actin disassembly in human neutrophils (Downey et al., 1990). However, when the cytosolic free Ca²⁺ level was buffered with MAPT (data not shown), sulfatides were still able to cause a marked decrease in the cellular F-actin responses induced by either β2-integrins or fMet-Leu-Phe (Table 1). Alternatively, a cellular increase in cAMP level, which can reverse the β2-integrin-induced F-actin response in electroporpermeabilized neutrophils (Löfgren et al., 1993), might explain the observed effect of sulfatides. This possibility was tested by pretreating the cells with Rp-cAMPS, a cAMP antagonist which inhibits the cAMP-induced activation of protein kinase A (Rodriquez-Aquilera et al., 1993). However, even in the presence of this cAMP antagonist, sulfatides could still impair the cellular F-actin responses induced by either β2-integrins or fMet-Leu-Phe (Table 1).

It has been reported that engagement of β2-integrins results in increased tyrosine kinase activity (Berton et al., 1994; Hellberg et al., 1995) and that tyrosine kinase activation is linked to agonist-induced modulations of the cytoskeleton (Chrzanowska-Wodnicka and Burridge, 1994; Hall and Ridley, 1994; Barry and Critchley, 1994) as well as integrin-directed activation of protein kinase A (Klemke et al., 1994). Consequently, we performed experiments to determine whether sulfatide-induced activation of L-selectin would interfere with the early β2-integrin-induced activation of tyrosine kinase(s). Engagement of L-selectin alone did not cause any apparent protein tyrosine phosphorylation (Fig. 7A, lane 2) in comparison with that seen upon activation of β2-integrins. The latter induced a rapid (30 seconds) tyrosine phosphorylation of several major proteins with molecular masses of about 60, 70,

![Fig. 7. (A) Effect of sulfatides on β2-integrin-induced protein tyrosine phosphorylation. Human neutrophils were incubated in medium alone (lanes 1 and 2) or with the mAb IB4 and PMA as described earlier (lanes 3, 4, and 5). Following incubation, the cells were washed and exposed to sulfatides (lanes 2 or 5) or medium alone (lanes 1, 3, and 4) for 1 minute before the addition of RAM for 30 seconds (lanes 4 and 5). The experimental treatment in short: lane 1, control with no additions; lane 2, sulfatides alone; lane 3, the mAb IB4 alone; lane 4, β2-integrin engagement; lane 5, exposure to sulfatides prior to β2-integrin engagement. The reactions were stopped by transferring the samples to an equal amount of ice-cold Ca²⁺ medium. The samples were subjected to gel electrophoresis and western blot analysis as described in Materials and Methods. The presence of tyrosine phosphorylated proteins was detected with an anti-phosphotyrosine mAb and a commercial ECL kit. The results shown are representative of at least 3 separate experiments. (B) Effect of sulfatides on β2-integrin-induced protein tyrosine phosphorylation in the absence of PMA. These results were obtained in an identical manner as described for those shown in A, except that the cells were pre-incubated in the absence of PMA. The results shown are representative of at least 4 separate experiments. (C) Effects of methyl-2,5-dihydroxycinnamate (erbstatin analogue) and pervanadate in the presence of sulfatides on the β2-integrin-induced F-actin response of neutrophils. The cells were incubated with erbstatin analogue (30 μg/ml; △), pervanadate (100 μM; ▼) or medium alone (●) for 45 minutes before the addition of mAb IB4 and PMA. The cells were washed and exposed to medium alone (●) or sulfatides (▼) for 1 minute prior to the addition of RAM. The cellular F-actin content at the indicated times was determined as described in Materials and Methods. Values are given as mean ± s.e.m (n=4) and expressed as a percentage of the time-zero value for each individual experiment.]
120 and 150 kDa (Fig. 7A, lane 4). However, if cells were pre-treated with sulfatides prior to engagement of their β2-integrins, there was a general reduction in the level of tyrosine phosphorylation, in particular, of the 60 and 150 kDa protein bands (Fig. 7A, lane 5). This effect of sulfatides on β2-integrin-induced protein tyrosine phosphorylation was not dependent upon pre-incubation with PMA, since similar results were obtained even in the absence of PMA (Fig. 7B, lanes 1-5). Thus, engagement of L-selectin inhibits or counteracts the β2-integrin-induced tyrosine kinase activity and in doing so may reduce the F-actin response elicited by β2-integrins.

We further tested the effect of methyl-2,5-dihydroxycinnamate, a stable analogue of the tyrosine kinase inhibitor erbstatin, on the β2-integrin-induced F-actin response. This analogue was chosen because it has been shown to inhibit the predominant tyrosine kinase in granulocytic cells (Ermould et al., 1993) and it is effective in blocking integrin actions (Williams et al., 1994). It is apparent that this tyrosine kinase inhibitor almost totally abolished the β2-integrin-induced F-actin response (Fig. 7C). However, we were unable to reverse the effect of sulfatides on the β2-integrin-induced F-actin response by pretreating cells with pervanadate, an inhibitor of tyrosine phosphatases (Fig. 7C). These results suggest that L-selectin activation impairs β2-integrin-induced F-actin formation by interfering with the β2-integrin signal transduction pathway at/or upstream of activation of a tyrosine kinase(s) rather than by increasing the activity of a cellular tyrosine phosphatase(s).

**DISCUSSION**

Neutrophils rolling along the activated endothelium of a vessel wall are loosely attached via their L-selectin to the endothelial cells as visualized by intravital microscopy (von Andrian et al., 1992). Despite such attachment, the neutrophils remain round in shape. The lack of a morphological response of neutrophils during their initial interaction with the endothelium is most likely a prerequisite for their rolling behaviour. The fact that neutrophils continue to roll if their subsequent interaction with integrins is prevented (von Andrian et al., 1991; Lawrence and Springer, 1991), suggests that the initial engagement of L-selectin on neutrophils does not have a major effect on the actin cytoskeleton. The present study confirms this observation by demonstrating that sulfatides do not cause an increase in cellular content of F-actin.

In addition to maintaining neutrophil rolling by decreasing the cellular content of F-actin, sulfatide-induced engagement of L-selectin was observed to impair the actin polymerizing capacity of β2-integrins. This was the case when sulfatides were given to the cells prior to antibody engagement of the β2-integrins and when they were given to neutrophils in continuous motion on an ICAM-1 coated surface. The same effect of sulfatides was also observed when an F-actin response was triggered by the chemotactic peptide fMet-Leu-Phe. In addition, the data demonstrating that both shedding and specific antibody blocking of L-selectin abolished the effect of sulfatides supports the notion that the observed effects are mediated via activation of L-selectin. Consequently, L-selectin is responsible not only for the rolling that precedes the firm β2-integrin-mediated adhesion of neutrophils to the endothelium, but also for a negative feed-back mechanism whereby firm adhesion is counteracted. This negative feed-back property of the L-selectin molecule could be responsible for directing the neutrophil to a specific site for firm adherence, i.e. where there is a relative dominance of ligands for β2-integrins, as compared to ligands for L-selectin. In addition, L-selectin engagement alone causes shedding of these adhesion molecules (Palecanda et al., 1992) which would also lead to a reduction in the negative feed-back action of L-selectin on β2-integrin-mediated adherence. Thus, it seems reasonable to conclude that such a shedding of L-selectin is the most likely explanation for the transient nature of the sulfatide-induced impairment of agonist-induced F-actin responses.

In leukocytes, a rise in both the cytosolic free Ca2+ concentration and intracellular cAMP levels has been found to counteract agonist-induced F-actin responses (Downey et al., 1990; Löfgren et al., 1993) and to inhibit motile events critically dependent on a proper F-actin response (Lew et al., 1985; Andersson et al., 1988). In our study, however, the effects of sulfatides on agonist-induced F-actin responses were not counteracted when a rise in the cytosolic free Ca2+ concentration was abolished, or when the activation of the cAMP-dependent protein kinase A was inhibited. Instead, we found that the ability of β2-integrin engagement to induce protein tyrosine phosphorylation was reduced by preexposure to sulfatides. The link between tyrosine kinase activation and cytoskeletal rearrangements (Chrzanowska-Wodnicka and Burridge, 1994; Hall and Ridley, 1994; Barry and Critchley, 1994) as well as integrin-directed cell motility (Klemke et al., 1994), suggests that β2-integrin-induced tyrosine phosphorylation is a possible target for the L-selectin-induced effect. This possibility is further implied by the observation that methyl-2,5-dihydroxycinnamate (a tyrosine kinase inhibitor) was able to block the β2-integrin-induced F-actin response. It seems reasonable to conclude that L-selectin engagement can affect the β2-integrin signal transduction pathway at/or upstream of its activation of a tyrosine kinase(s) or indirectly by increasing the cellular activity of one or more tyrosine phosphatases. The present observation that the effect of sulfatides on the β2-integrin-induced F-actin response could not be counteracted by pretreating the cells with pervanadate seems to refute the latter possibility.

Adhesion of neutrophils to purified P-selectin, binding to all sialyl Lewis x epitopes, has been shown to potentiate β2-integrin functions (Cooper et al., 1994). However, of more specific interest for the present study is the observations that specific antibody cross-linking of L-selectin on human neutrophils enhanced both the β2-integrin adhesiveness (Simon et al., 1995) as well as a subsequent oxidative burst induced by N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMet-Leu-Phe) or TNFα (Waddell et al., 1994). These observations and the fact that the potentiating effect of L-selectin engagement on agonist-induced oxidative burst is mediated by a rise in the cytosolic free Ca2+ level (Waddell et al., 1994) whereas its effect on agonist-induced F-actin responses are not, suggest that engagement L-selectins can have distinct effects on different neutrophil functions.
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(Received 22 November 1995 - Accepted, in revised form, 11 June 1996)