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Calmodulin Regulates L-Selectin Adhesion Molecule Expression and Function through a Protease-Dependent Mechanism

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Summary

Expression of the L-selectin adhesion molecule is rapidly down-regulated upon cell activation through proteolysis at a membrane-proximal site. Here we demonstrate that calmodulin, an intracellular calcium regulatory protein, specifically coprecipitates with L-selectin through a direct association with the cytoplasmic domain of L-selectin. Furthermore, calmodulin inhibitors disrupt L-selectin-dependent adhesion by inducing proteolytic release of L-selectin from the cell surface. The effects of the calmodulin inhibitors on L-selectin expression and function can be prevented by cotreatment with a hydroxamic acid–based metalloprotease inhibitor. Our results suggest a novel role for calmodulin in regulating the expression and function of an integral membrane protein through a protease-dependent mechanism. These findings may have broader implications for other cell surface proteins that also undergo regulated proteolysis.

Introduction

L-selectin (CD62L) is a leukocyte cell surface adhesion molecule involved in recruitment of leukocytes to sites of inflammation and in homing of naive lymphocytes to peripheral lymphoid tissue. L-selectin belongs to a family of C-type lectin adhesion molecules that also includes E-selectin (CD62E) and P-selectin (CD62P), which are expressed on activated endothelial cells and, in the case of P-selectin, on activated platelets. The selectins participate in the initial events in leukocyte binding to vascular endothelial cells by mediating, in part, leukocyte rolling under conditions of hydrodynamic shear stress (reviewed in Butcher, 1991; Springer, 1994). Neutrophil–neutrophil interactions under flow conditions also involve L-selectin (Bargatze et al., 1994; Alon et al., 1996; Walcheck et al., 1996b). The ligands for L-selectin on high endothelial venules are broadly defined by the MECA-79 MAb (Streeter et al., 1988). Specific ligands include GlyCAM-1 (Lasky et al., 1992) and CD34 (Baumhueter et al., 1993), which bear complex sialylated, fucosylated, and sulfated carbohydrates (Hemmerich et al., 1994). PSGL-1, a P-selectin ligand on leukocytes, can also bind to L-selectin during neutrophil–neutrophil interactions (Guyer et al., 1996; Spertini et al., 1996; Tu et al., 1996; Walcheck et al., 1996b). L-selectin is distributed on the tips of microvilli projections, a location that presumably facilitates rolling interactions (Picker et al., 1991; von Andrian et al., 1995). L-selectin adhesiveness can be transiently increased by cell activation (Spertini et al., 1991). Recently L-selectin has been shown to transduce signals leading to calcium flux, protein phosphorylation, enhanced oxidative burst, and activation of β1 and β2 integrins (Laudanna et al., 1994; Crockett-Torabi et al., 1995; Waddell et al., 1994, 1995; Simon et al., 1995; Brenner et al., 1996; Hwang et al., 1996; Steeber et al., 1997). L-selectin cell surface expression is rapidly down-regulated by a proteolytic mechanism that cleaves L-selectin at a membrane-proximal site upon cell activation or upon L-selectin cross-linking (Kishimoto et al., 1989; Palecanda et al., 1992; Kahn et al., 1994; Chen et al., 1995; Migaki et al., 1995). Inhibition of L-selectin shedding by hydroxamic acid–based metalloprotease inhibitors (Feehan et al., 1996; Preece et al., 1996) alters the L-selectin-dependent component of neutrophil rolling on MECA-79 antigen (Walcheck et al., 1996a) and neutrophil aggregation (Bennett et al., 1996), but does not affect neutrophil rolling on or transmigration through IL-1-stimulated endothelial cells, which support multiple adhesion pathways (Allport et al., 1997).

The cytoplasmic domain of L-selectin is relatively small (17 amino acids) but essential for normal L-selectin function (Kansas et al., 1993). Deletion of the COOH-terminal 11 amino acids of the L-selectin cytoplasmic tail has a profound effect on L-selectin-dependent adhesion, including inhibition of neutrophil rolling in vivo and inhibition of the ex vivo binding of lymphocytes to high endothelial venules in frozen thin sections of peripheral lymph node tissue (Kansas et al., 1993). Cytoskeletal involvement in adhesion was implicated based upon inhibition of L-selectin function by cytochalasin B, a microfilament-disrupting agent (Kansas et al., 1993), and the finding that α-actinin can interact with the cytoplasmic domain of L-selectin (Pavalko et al., 1995). Recently Haribabu et al. (1997) have demonstrated that the cytoplasmic tail of L-selectin is phosphorylated on serine upon cell activation with phorbol esters or with chemotactic agents.

In this report, we demonstrate that calmodulin, a calcium regulatory protein, is specifically coprecipitated with L-selectin. Moreover, purified calmodulin binds directly to the cytoplasmic tail of L-selectin. Calmodulin inhibitors, such as trifluoperazine, inhibit L-selectin-dependent neutrophil rolling on MECA-79 antigen and lymphocyte binding to peripheral lymph node high endothelial venules. These calmodulin inhibitors directly induce proteolytic shedding of L-selectin without causing general cell activation. The expression of L-selectin and the rolling adhesions of trifluoperazine-treated neutrophils can be rescued by cotreatment with a hydroxamic acid–based metalloprotease inhibitor that inhibits L-selectin shedding.

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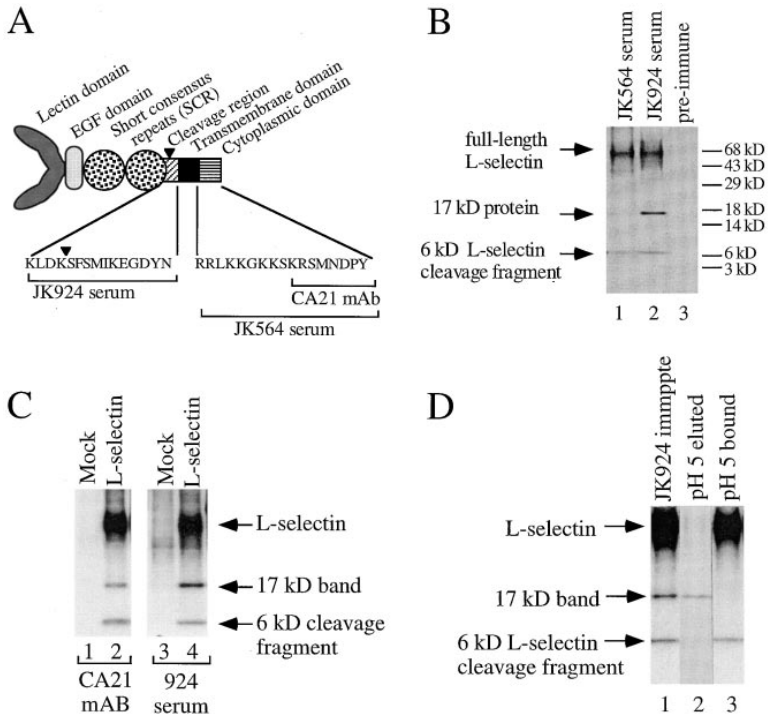

Figure 1. A 17 kDa Band Coprecipitates with L-Selectin

(A) Schematic representation of the L-selectin adhesion molecule. The amino acid sequence of the extracellular cleavage region (diagonally hatched) and the cytoplasmic domain (striped) is shown below. The predicted cleavage site is indicated by the inverted triangle. The epitope(s) recognized by the JK924 serum, directed against the cleavage region; the JK564 serum, directed against the entire cytoplasmic domain; and the CA21 MAb, directed against the COOH-terminal 8 amino acid residues of the cytoplasmic tail, are indicated.

(B) JK924 versus JK564 immunoprecipitations. L-selectin-transfected COS cells were metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with JK564 anti-cytoplasmic domain serum (lane 1), JK924 anti–cleavage region serum (lane 2), or preimmune serum (lane 3). Samples were washed and subjected to SDS-PAGE, followed by autoradiography with fluorography. The full-length membrane-associated L-selectin, the 6 kDa cleavage fragment of L-selectin, and the 17 kDa band are indicated with arrows. Migration of molecular weight standards is indicated.

(C) The 17 kDa protein coprecipitates with CA21 MAb and JK924 serum. Metabolically labeled L-selectin transfectants (lanes 2 and 4) or mock transfectants (lanes 1 and 3) were lysed and immunoprecipitated with CA21 anti-cytoplasmic tail MAb (lanes 1 and 2) or with the JK924 anti–cleavage region serum (lanes 3 and 4).

(D) The 17 kDa protein elutes from JK924 precipitates with a pH 5 wash. Metabolically labeled L-selectin transfectants were lysed and immunoprecipitated with the JK924 anti–cleavage region serum and protein A-Sepharose beads (lane 1). Duplicate sets of JK924 beads were subjected to a pH 5 wash prior to elution with SDS-PAGE sample buffer. Proteins eluted with the pH 5 wash were retained and subjected to SDS-PAGE (lane 2). Proteins still bound to the JK924 beads were eluted with SDS-PAGE sample buffer (lane 3).

Results

Coprecipitation of Calmodulin with L-Selectin

We have previously shown that L-selectin is proteolytically cleaved at a membrane proximal site, resulting in the formation of a 68 kDa soluble form of L-selectin and a 6 kDa transmembrane fragment (Kahn et al., 1994). In the course of our studies on L-selectin proteolysis, we observed a 17 kDa protein that coprecipitated with L-selectin from [^{35}S]methionine biosynthetically labeled L-selectin transfectants using the JK924 antiserum directed against the 15 amino acid extracellular cleavage region of L-selectin (Figures 1A and 1B, and Kahn et al., 1994). The 17 kDa protein was not observed in immunoprecipitates with the JK564 serum directed against the entire 17 amino acid sequence of the L-selectin cytoplasmic tail (Figures 1A and 1B). However, the CA21 MAb directed against the COOH-terminal 8 amino acid residues of the L-selectin cytoplasmic tail also coprecipitated a 17 kDa protein, which comigrated precisely with the 17 kDa protein observed in JK924 immunoprecipitates (Figures 1A and 1C). The 17 kDa protein was not observed in JK924 or CA21 immunoprecipitates from mock transfected cells, suggesting that the association of the 17 kDa protein is specific for L-selectin (Figure 1C). The 17 kDa protein could be eluted from JK924 immunoprecipitates with a pH5 wash (Figure 1D), suggesting that the 17 kDa protein is not directly bound by the JK924 antiserum. Similar results were obtained with pH 5 elution of CA21 MAb immunoprecipitates (data not shown).

To determine whether the 17 kDa protein was a cleavage product of L-selectin, we attempted to perform Western blot analysis on CA21 immunoprecipitates with antibodies directed against the lectin domain, cytoplasmic domain, and the membrane-proximal cleavage site of L-selectin. However, the 17 kDa protein displayed an unusual property of washing off the PVDF membrane, an observation that was confirmed by using metabolically labeled material and exposing the blot to film before and after the Western blotting procedure. While the radiolabeled full-length L-selectin and the 6 kDa transmembrane cleavage product of L-selectin were retained on the membrane, the 17 kDa band was selectively lost during the incubation and wash steps (data not shown). This observation was reminiscent of a report describing the immunoblotting of calmodulin, a 17 kDa calcium regulatory protein. Van Eldik and Wolchok (1984) described a method for fixing the blot with glutaraldehyde in order to retain calmodulin on the blot. This procedure also worked in our hands to retain the 17 kDa band on Western blots of CA21 immunoprecipitates. The 17 kDa band did not react with any of the anti-L-selectin monoclonal or polyclonal antibodies (Figure 2A and data not shown). However, the 17 kDa protein, which coprecipitated with L-selectin from both CA21 (anti-L-selectin cytoplasmic tail) immunoprecipitates and JK924 (anti-L-selectin cleavage region) immunoprecipitates,

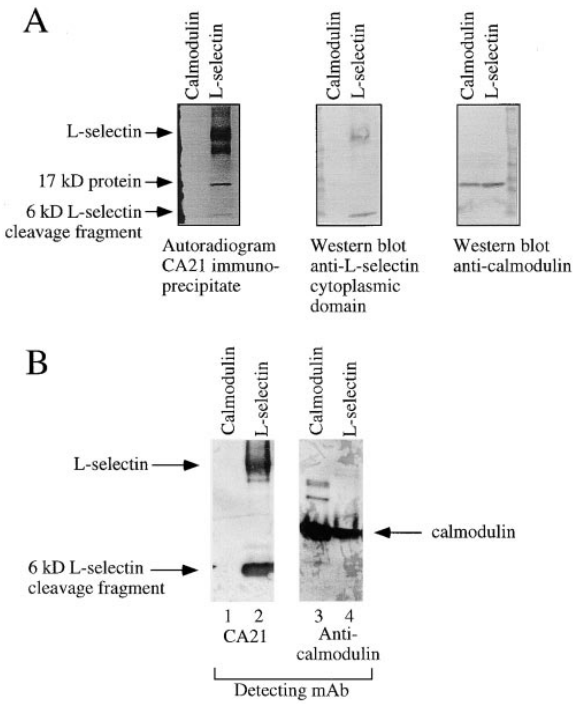

Figure 2. Identification of the 17 kDa Band as Calmodulin

(A) Western blotting of CA21 immunoprecipitates. L-selectin-transfected COS cells were metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with CA21 MAb. The CA21 immunoprecipitate was divided among two lanes of an SDS-PAGE gel. A purified calmodulin standard was run in adjacent lanes, as indicated. The gel was transferred to a PVDF membrane and divided in half. The blots were subjected to autoradiography (left panel) to visualize the migration of the full-length membrane-associated L-selectin, the 6 kDa cleavage fragment of L-selectin, and the 17 kDa band, as indicated. One blot was then subjected to Western blot analysis with the CA21 anti-cytoplasmic domain MAb (middle panel). The duplicate blot was subjected to Western blot analysis with an anti-calmodulin MAb (right panel). The blots were reacted with anti-mouse IgG antibodies conjugated with alkaline phosphatase and then developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride.

(B) Coprecipitation of calmodulin from L-selectin transfected L1.2 lymphoid cells. L-selectin L1.2 cells were lysed and immunoprecipitated with the CA21 anti-cytoplasmic tail MAb and run on duplicate lanes of an SDS-PAGE gel (lanes 2 and 4). Purified calmodulin was run in adjacent lanes (lanes 1 and 3). The gel was transferred to PVDF membrane and divided in half. One blot was subjected to Western blot analysis with the CA21 MAb (lanes 1 and 2). The duplicate blot was subjected to Western blot analysis with an anti-calmodulin MAb (lanes 3 and 4).

comigrated with purified calmodulin and reacted with an anti-calmodulin MAb (Figure 2A and data not shown). Calmodulin was also coprecipitated with L-selectin from L-selectin-transfected L1.2 lymphoid cells (Figure 2B).

Direct Binding of Calmodulin to the Cytoplasmic Tail of L-Selectin

Calmodulin is an intracellular regulatory protein. If the interaction of calmodulin with L-selectin is physiologically relevant, then calmodulin should bind selectively to the cytoplasmic domain of L-selectin. Purified biotinylated calmodulin bound directly to a purified preparation of full-length L-selectin but did not bind to a purified

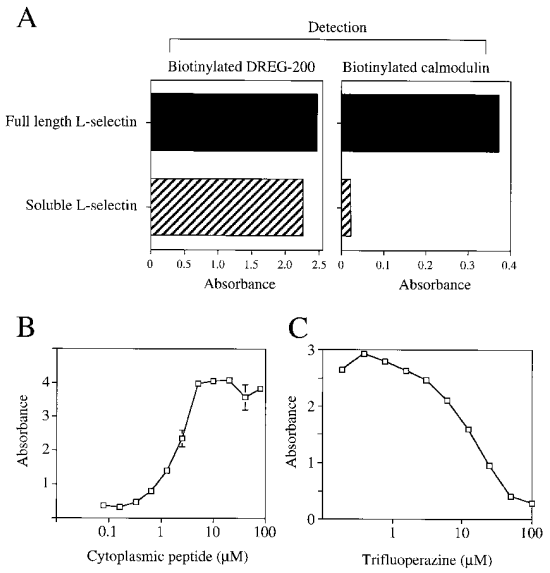

Figure 3. Calmodulin Binds Directly to the Cytoplasmic Tail of L-Selectin

(A) Calmodulin binds full-length but not soluble L-selectin. Full-length membrane-associated L-selectin was purified from L-selectin transfectants with DREG-200 MAb. A recombinant soluble form of L-selectin lacking the transmembrane and cytoplasmic domains was produced and purified as previously described (Migaki et al., 1995). The full-length (closed bars) and soluble (hatched bars) L-selectin was trapped with the DREG-55 anti-L-selectin MAb. The presence of L-selectin was confirmed by ELISA detection with biotinylated DREG-200 MAb, which reacts with both the full-length and soluble L-selectin (left panel). Binding of calmodulin to L-selectin was assessed with biotinylated calmodulin (right panel).

(B) Direct binding of calmodulin to the L-selectin cytoplasmic tail. Purified calmodulin was absorbed to the bottom of microtiter wells. A biotinylated peptide corresponding to the entire amino acid sequence of the L-selectin cytoplasmic domain was titrated at various concentrations, as indicated. Binding of the peptide was visualized by addition of streptavidin-horseradish peroxidase and 2,2′-azinobis (3-ethylbenzthioazolinesulfonic acid) substrate.

(C) Binding of calmodulin to the L-selectin cytoplasmic tail is inhibited by trifluoperazine. The biotinylated L-selectin cytoplasmic tail peptide was incubated with immobilized calmodulin. Trifluoperazine was titrated in at various concentrations, as indicated.

recombinant soluble (extracellular) fragment of L-selectin, which lacked the transmembrane and cytoplasmic domains (Figure 3A). Moreover, a biotinylated synthetic peptide corresponding to the 17 amino acid sequence of the L-selectin cytoplasmic tail bound directly to purified calmodulin. The binding of calmodulin to the cytoplasmic tail peptide was dose dependent and saturable (Figure 3B), and blocked by trifluoperazine, a calmodulin inhibitor (Figure 3C). Blocking with trifluoperazine had an IC50 of about 4 μM, a dose range that is consistent with trifluoperazine inhibition of other calmodulin-dependent interactions (Jones and McCord, 1984). Calmodulin binding domains are characteristically amphipathic helices that are rich in basic residues (O'Neil and DeGrado, 1990). The L-selectin cytoplasmic domain, NH_2-**RR**L**KK** G**KK**S**KR**SMNDPY-COOH, has an unusual motif of dibasic residues (basic residues are shown in boldface) of which the first nine residues are completely conserved across human, mouse, and rat sequences. A helical

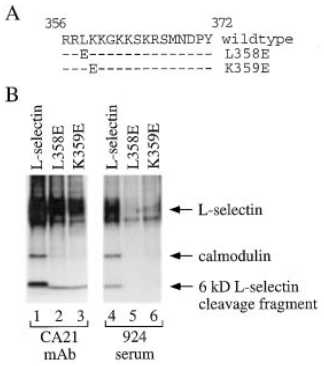

Figure 4. Point Mutations in the Cytoplasmic Tail Disrupt Calmodulin Binding and Reactivity with the JK924 Anti–Cleavage Domain Serum

(A) Amino acid sequence of the wild-type and mutant L-selectin cytoplasmic tail. The cDNA encoding full-length L-selectin was mutated in the cytoplasmic tail, changing leucine at position 358 to glutamic acid (L358E) or changing lysine at position 359 to glutamic acid (K359E).

(B) COS cells were transfected with wild-type L-selectin (lanes 1 and 4), the L358E L-selectin cytoplasmic tail mutant (lanes 2 and 5), or the K359E L-selectin cytoplasmic tail mutant (lanes 3 and 6). Transfectants were metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with either the CA21 anti-cytoplasmic tail MAb (lanes 1–3) or the JK924 anti–cleavage region serum (lanes 4–6). Samples were subjected to SDS-PAGE and autoradiography.

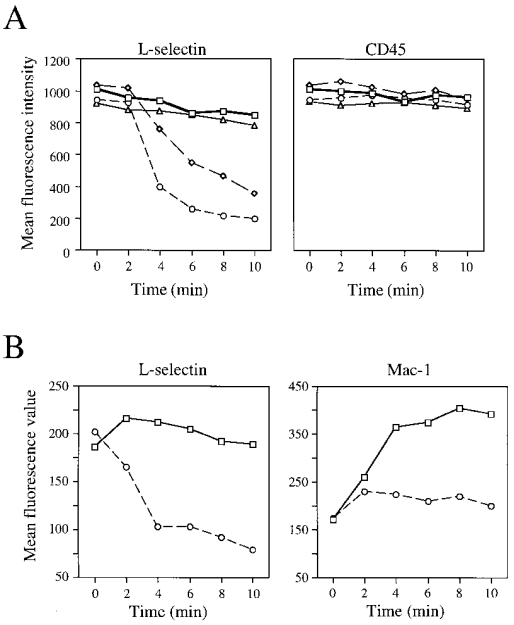

Figure 5. Calmodulin Inhibitors Induce L-Selectin Down-Regulation

(A) Calmidazolium enhances fMLP-induced shedding of L-selectin. Neutrophils were treated with DMSO control at 37°C (square symbols, thick line); or with 5×10^{-7} M fMLP (diamond symbols, long dashed line); or with 5 X 10^{-7} M fMLP plus 5 μM calmidazolium (circle symbols, short dashed line); or with 5×10^{-7} M fMLP, 5 μM calmidazolium, and 25 μM KD-IX-73-4 (triangle symbols, solid thin line). L-selectin expression and CD45 expression were monitored in real time by quantitative flow cytometry every 2 min with phycoerythrin-conjugated anti-L-selectin MAb (left panel) and fluorescein-conjugated anti-CD45 MAb (right panel), respectively. Ten thousand cells were analyzed at each timepoint, and data are reported as the mean fluorescence value at each timepoint. Data are representative of at least three similar experiments.

(B) Trifluoperazine induces L-selectin shedding in the absence of cell activation. Neutrophils were treated with DMSO control at 37°C (square symbols, solid line) or treated with 20 μM trifluoperazine (circle symbols, dashed line). L-selectin expression and Mac-1 expression were monitored in real time by quantitative flow cytometry every 2 min with phycoerythrin-conjugated anti-L-selectin MAb (left panel) and fluorescein-conjugated anti-Mac-1 MAb (right panel), respectively. Data are representative of at least three similar experiments.

model of the L-selectin cytoplasmic tail peptide shows a basic face similar to that seen with other calmodulin-binding proteins (data not shown). The CA21 MAb epitope has been mapped to the COOH-terminal 8 residues of the cytoplasmic domain (data not shown), which falls outside of this putative calmodulin-binding region. In contrast, rabbit polyclonal antiserum JK564 raised against the entire cytoplasmic tail of L-selectin did not coprecipitate calmodulin (Figures 1A and 1B). The JK564 polyclonal serum but not CA21 MAb blocked binding of the cytoplasmic tail peptide to purified calmodulin, suggesting that the JK564 antiserum binds, at least in part, to the calmodulin binding site (data not shown).

We next mutated specific amino acids within the putative calmodulin-binding site of L-selectin (Figure 4A). Lys359Glu disrupts the basic charge face but retains the predicted amphihelical nature of the cytoplasmic tail, and Leu358Glu disrupts the hydrophobic face. Both mutants were expressed on the cell surface at a level similar to wild-type transfectants, as judged by fluorescence microscopy and by flow cytometry (data not shown). Metabolic labeling of COS cells transfected with these mutants and immunoprecipitation with the CA21 MAb showed that the coprecipitation of calmodulin was profoundly reduced compared to wild-type L-selectin transfectants (Figure 4B, left panel). Interestingly, these mutations in the cytoplasmic tail affected the reactivity of the mutant L-selectin molecules with the JK924 serum directed against the extracellular cleavage site of L-selectin (Figure 4B, right panel).

Calmodulin Inhibitors Induce L-Selectin Down-Regulation

We next examined whether calmodulin inhibitors affected L-selectin cell surface expression. L-selectin is known to be rapidly shed within minutes following neutrophil activation with chemotactic factors (Kishimoto et al., 1989). Treatment of neutrophils with 5 μM calmidazolium, a calmodulin inhibitor, further accelerated the rate of L-selectin down-regulation upon stimulation with fMLP as judged by flow cytometry (Figure 5A). Expression of CD45 was not affected by calmidizolium (Figure 5A). Similar results were obtained with trifluoperazine and W7 [N-(-6-aminohexyl)-5-chloro-1-napthalenesulfonamide], two other well-characterized calmodulin inhibitors. W7 enhanced L-selectin shedding, while the structurally related but less potent calmodulin inhibitor W5 [N-(-6-aminohexyl)-1-napthalenesulfonamide] had no effect on L-selectin expression at similar doses (data not shown).

We next discovered that trifluoperazine, in the absence of any activating agent, was sufficient to induce down-regulation of L-selectin. Trifluoperazine-induced shedding of L-selectin from neutrophils was rapid (Figure 5B), following kinetics similar to those seen with

neutrophil activation with chemotactic factors (Kishimoto et al., 1989). Trifluoperazine also induced L-selectin shedding on peripheral blood lymphocytes. The kinetics of lymphocyte L-selectin shedding were slower than that observed with trifluoperazine-treated neutrophils but similar to that observed with phorbol ester–activated lymphocytes (data not shown). Trifluoperazine did not cause general neutrophil activation as judged by the lack of Mac-1 up-regulation. Indeed, trifluoperazine inhibited the spontaneous up-regulation of Mac-1 that is observed in some preparations of neutrophils incubated at 37°C (Figure 5B). In fact, inhibition of calmodulin function is generally associated with the inhibition of cell activation and has previously been shown to inhibit activation-induced neutrophil Mac-1 up-regulation, oxidative burst, and secondary granule release (Alobaidi et al., 1981; Smith et al., 1981; Elferink et al., 1982; Berger et al., 1985; Wright and Hoffman, 1987). Trifluoperazine or calmidazolium-treatment did not alter expression of other neutrophil cell surface markers, such as CD45 and PSGL-1 (Figure 5A and data not shown).

Calmodulin Inhibitors Regulate L-Selectin Expression through a Proteolytic Mechanism

We and others have recently shown that hydroxamic acid–based metalloprotease inhibitors inhibit the activation-induced shedding of L-selectin (Feehan et al., 1996; Preece et al., 1996). L-selectin shedding induced by either fMLP alone or fMLP plus calmidazolium was blocked by the KD-IX-73-4 hydroxamic acid–based metalloprotease inhibitor (Figure 5A). The trifluoperazine-induced shedding of L-selectin was also inhibited by cotreatment with the KD-IX-73-4 inhibitor (Figure 6A). Activation-induced shedding of L-selectin is known to occur through a proteolytic mechanism that cleaves L-selectin at a membrane-proximal site to yield a 6 kDa transmembrane fragment. Immunoprecipitation analysis of the 6 kDa transmembrane cleavage product of L-selectin confirmed that trifluoperazine induced shedding through a proteolytic mechanism (Figure 6B). Moreover, the KD-IX-73-4 hydroxamic acid–based metalloprotease inhibitor blocked the trifluoperazine-induced proteolysis of L-selectin (Figure 6B). These results suggested that trifluoperazine-induced shedding occurs through a proteolytic pathway similar to that observed with cell activation induced with fMLP and other neutrophil chemotactic agents.

L-Selectin-Dependent Adhesion Is Disrupted by Calmodulin Inhibitors

To assess whether L-selectin function is modulated by calmodulin, calmodulin inhibitors were tested for their effect on L-selectin-mediated adhesion. We examined lymphocyte binding to high endothelial venules (HEV) in frozen thin sections of peripheral lymph node tissue. This interaction is dependent upon L-selectin interacting with one or more ligands expressed by the HEV. Lymphocyte adhesion to HEV was blocked by trifluoperazine (Figure 7A).

We also examined neutrophil rolling on purified MECA-79 antigen, a physiological L-selectin ligand, under hydrodynamic flow conditions. This rolling interaction is completely inhibited by antibodies directed against

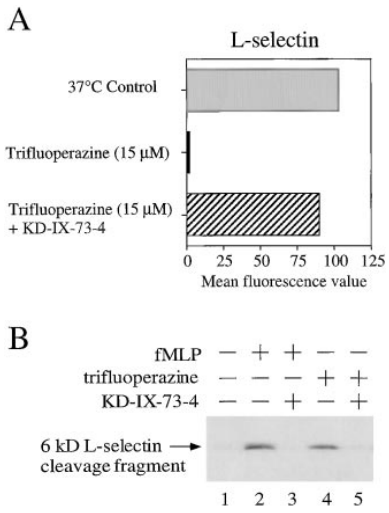

Figure 6. Calmodulin Inhibitors Induce L-Selectin Shedding through a Protease-Dependent Mechanism

(A) Neutrophils were treated with DMSO carrier (gray bar), with 15 μM trifluoperazine (closed bar), or with 15 μM trifluoperazine plus 25 μM KD-IX-73-4 for 15 min at 37°C. Cells were stained for L-selectin expression with phycoerythrin-conjugated Leu-8 MAb and analyzed by flow cytometry. Data are representative of at least three similar experiments.

(B) Analysis of the 6 kDa cleavage fragment of L-selectin. Neutrophils were treated with DMSO carrier (lane 1), with 5×10^{-7} M fMLP (lane 2), with 5×10^{-7} M fMLP plus 25 μM KD-IX-73-4 (lane 3), with 15 μM trifluoperazine (lane 4), or with 15 μM trifluoperazine plus 25 μM KD-IX-73-4 (lane 5) for 15 min at 37°C. Cells were lysed and immunoprecipitated with CA21 anti-L-selectin cytoplasmic tail MAb coupled to Sepharose beads. Immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membranes. The 6 kDa cleavage fragment of L-selectin was visualized by Western blot analysis with biotinylated CA21 MAb.

L-selectin (Lawrence et al., 1995; Finger et al., 1996; unpublished data). Trifluoperazine, a potent inhibitor of calmodulin, significantly reduced neutrophil rolling on MECA-79 antigen (Figure 7B). Trifluoperazine at 10–25 μM was not toxic to the cells and did not grossly alter neutrophil morphology, as judged by light and by transmission electron microscopy (unpublished data).

We next examined whether cotreatment with the KD-IX-73-4 protease inhibitor would be sufficient to rescue the ability of trifluoperazine-treated neutrophils to roll on MECA-79 antigen. Neutrophils were treated with 15 μM trifluoperazine in the presence or absence of 25 μM KD-IX-73-4 and introduced into a MECA-79 antigen-coated flow chamber at a wall shear stress of 1.5 dynes/cm^2. Cells treated with trifluoperazine alone were unable to establish a rolling interaction. However, cotreatment with the KD-IX-73-4 metalloprotease inhibitor rescued the ability of trifluoperazine-treated cells to form rolling interactions with the MECA-79 antigen surface (Figure 7C).

Discussion

Calmodulin is a ubiquitous calcium-binding protein that can act as a cofactor for many cytosolic enzymes, influence cytoskeletal architecture, and regulate function of integral membrane proteins (Crivici and Ikura, 1995; Gnegy, 1995). We demonstrate that calmodulin inhibitors, such as trifluoperazine and calmidazolium, inhibit

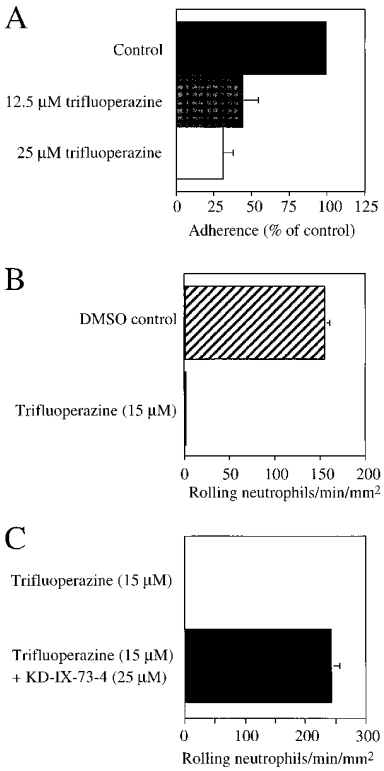

Figure 7. Calmodulin Inhibitors Affect L-Selectin-Dependent Adhesion

(A) Trifluoperazine inhibits lymphocyte binding to high endothelial venules. Peripheral blood lymphocytes were allowed to adhere to frozen thin sections of peripheral lymph node tissue at 7°C, as previously described (Kishimoto et al., 1990). Lymphocytes were treated with DMSO carrier (closed bar), with 12.5 μM trifluoperazine (gray bar), or with 25 μM trifluoperazine (open bar). Lymphocytes bound per high endothelial venule were counted. Data are expressed as adherence to a percent of the DMSO control.

(B) Trifluoperazine inhibits neutrophil rolling on MECA-79 antigen. A parallel plate flow chamber was coated with purified MECA-79 antigen, a ligand for L-selectin, as previously described (Walcheck et al., 1996a). Neutrophils were treated with DMSO carrier (hatched bar) or with 15 μM trifluoperazine (closed bar) for 10 min at 37°C before infusion into the flow chamber. Neutrophils were allowed to establish a rolling interaction with the MECA-79 antigen at a shear stress of 1.5 dynes/cm^2. Data are expressed as the number of neutrophils rolling/min/mm^2.

(C) KD-IX-73-4 protease inhibitor rescues rolling of trifluoperazine-treated cells. Neutrophils were treated with 15 μM trifluoperazine or with 15 μM trifluoperazine plus 25 μM KD-IX-73-4 (closed bar) for 10 min at 37°C before infusion into the flow chamber.

L-selectin function by inducing L-selectin down-regulation. L-selectin expression and function can be rescued in trifluoperazine-treated neutrophils by cotreatment with a hydroxamic acid–based inhibitor of the L-selectin protease. These results suggest a novel role for calmodulin in regulating surface expression of an integral membrane protein through a proteolytic mechanism. It is possible that the action of the calmodulin inhibitors on L-selectin expression occurs at a level downstream of L-selectin itself, perhaps at the level of the protease or a regulatory protein. Another possible confounding factor is that calmodulin inhibitors may nonspecifically affect membrane fluidity, which could alter L-selectin shedding indirectly. However, the observation that calmodulin coprecipitates with L-selectin and that purified calmodulin binds directly to the cytoplasmic tail of the L-selectin raises the possibility that calmodulin may regulate L-selectin expression and function through a direct interaction with the cytoplasmic domain of L-selectin.

Calmodulin usually acts as a calcium switch, binding to target sequences upon calcium flux following cell activation. An unusual feature of the apparent regulation of L-selectin expression by calmodulin is that calmodulin inhibitors induce L-selectin shedding in the absence of neutrophil activation. A model that is consistent with our current data would suggest that calmodulin is already bound to the L-selectin cytoplasmic tail in the resting cell and that removal of calmodulin through cell activation, or experimentally with trifluoperazine, results in L-selectin shedding. A role for calmodulin in resting cells is not unprecedented, as calmodulin has been shown to bind a number of target sequences in unactivated cells. Some target proteins, such as neuromodulin, neurogranin, and myosin I, bind to calmodulin with higher affinity in the absence of calcium than in the presence of calcium (Alexander et al., 1987; Swanljung-Collins and Collins, 1991; Huang et al., 1993; Crivici and Ikura, 1995; Gnegy, 1995). In the case of neuromodulin, an influx of calcium is thought to induce release of calmodulin from neuromodulin into the cytosol (Alexander et al., 1987). The brush border myosin I protein is thought to bind three or four calmodulin molecules with low Ca^{2+} affinity. Additional Ca^{2+} induces dissociation of one or more calmodulin molecules, resulting in altered myosin I function (Swanljung-Collins and Collins, 1991). Other targets, such as the adipocyte p170 protein (McDonald and Lawrence, 1989) and chick lens gap junction protein (Welsh et al., 1982), bind calmodulin in the absence of calcium. The requirement for calcium is not absolute for calmodulin binding to L-selectin in vitro. Purified calmodulin binds to the cytoplasmic tail of L-selectin even in the presence of EGTA (unpublished data). However, it is possible that low levels of calcium may potentiate binding interaction. The precise requirement for calcium in calmodulin interactions with L-selectin in cells will require more detailed studies.

Calmodulin is involved in many fundamental signaling pathways, activating the function of various kinases (CaM kinase I and II, myosin light chain kinase), phosphatases (calcineurin), ion channels (plasma membrane Ca^{2+} pump), and other cytosolic enzymes, such as phosphodiesterase, adenylate cyclase, and nitric oxide synthetase (Crivici and Ikura, 1995; Gnegy, 1995). Thus, it is not surprising that inhibitors of calmodulin typically inhibit cell activation processes. Calmodulin inhibitors have been shown to inhibit neutrophil oxidative burst, Mac-1 up-regulation, secondary granule release, cell migration, and motility (Alobaidi et al., 1981; Smith et al., 1981; Elferink et al., 1982; Berger et al., 1985; Wright and Hoffman, 1987). The shedding of L-selectin is generally regarded as a hallmark of neutrophil activation (Kishimoto et al., 1989). Paradoxically, we have found that calmodulin inhibitors accelerate L-selectin shedding in the presence of activating factors and can induce shedding even in the absence of overt cell activation. One

possible explanation is that the L-selectin protease requires active repression mediated by calmodulin in order to maintain L-selectin expression. The addition of calmodulin inhibitors may allow for removal of an active repressor, allowing shedding to occur as a default mechanism. For example, release of calmodulin from the cytoplasmic tail of L-selectin could induce a conformational change that exposes the extracellular cleavage site to a constitutively active protease. Similar "inside-out" signaling mechanisms have been proposed to regulate conformational changes in the ligand binding sites of integrins (Dustin and Springer, 1989) and of L-selectin itself (Spertini et al., 1991).

Interestingly, epitopes on the cytoplasmic tail and the extracellular membrane proximal cleavage region of L-selectin do appear to be functionally linked. Steeber et al. (1997) have recently shown that mutations in the extracellular membrane-proximal cleavage site of L-selectin that inhibit its shedding also have a profound effect on intracellular signaling through L-selectin. Thus, shedding of L-selectin and signaling through L-selectin may be intimately linked. In this report, we find that coprecipitation of calmodulin with L-selectin is most evident with the CA21 MAb directed against the cytoplasmic tail and with the JK924 serum directed against the extracellular cleavage site. Moreover, mutations in the L-selectin cytoplasmic tail that disrupt calmodulin binding also disrupt recognition of L-selectin by the JK924 anti–cleavage site serum. Thus, mutations in the cytoplasmic domain appear to affect conformation of an extracellular epitope. Binding by calmodulin may influence the presentation of the cleavage region and its accessibility to the JK924 serum (or to the natural L-selectin protease). These results would be consistent with our findings that calmodulin inhibitors, which act intracellularly, can induce L-selectin shedding through proteolysis at an extracellular cleavage site. Interestingly, function-blocking anti-L-selectin MAbs, such as the DREG MAb series, do not coprecipitate calmodulin efficiently. Recently, binding of the DREG MAbs (Waddell et al., 1995; Steeber et al., 1997) and other function-blocking MAbs (Steeber et al., 1997), in the absence of cross-linking, have been shown to induce signaling through L-selectin. It is possible that binding of MAbs to the lectin domain mimics ligand binding and induces a conformational change that causes calmodulin dissociation, leading to subsequent signaling events and cleavage of L-selectin.

A common mechanism to regulate calmodulin binding negatively is through serine phosphorylation of the target protein at sites in or near the calmodulin-binding region. Proteins such as MARCKS, neurogranin, and neuromodulin contain critical serine residues, which upon phosphorylation inhibit calmodulin binding (Alexander et al., 1987; Hartwig et al., 1992; Huang et al., 1993; Crivici and Ikura, 1995; Gnegy, 1995). Recently Haribabu et al. (1997) have demonstrated that L-selectin becomes phosphorylated on serine upon activation of L-selectin transfectants with phorbol esters or with chemoattractants. Phosphorylation of Ser-364, a residue conserved across species, would be predicted to disrupt the putative calmodulin-binding site of L-selectin. An alternative mechanism may be that a calcium flux following cell activation may expose more abundant and higher affinity binding sites for calmodulin. The calcium influx will also induce a conformational change in calmodulin (Crivici and Ikura, 1995; Gnegy, 1995), perhaps causing calmodulin to dissociate from the cytoplasmic tail of L-selectin. In this regard, it is interesting to note that cross-linking of L-selectin is sufficient to induce a calcium flux (Laudanna et al., 1994; Crockett-Torabi et al., 1995; Waddell et al., 1995) and to induce shedding of L-selectin (Palecanda et al., 1992) in the absence of any overt activating signal. Recently C-reactive protein (CRP), an acute phase protein, has been shown to induce L-selectin shedding in the absence of cell activation (Zouki et al., 1997). It will be of interest to determine if CRP-induced shedding of L-selectin involves calmodulin.

Deletion of the COOH-terminal 11 amino acid residues of the L-selectin cytoplasmic tail has been shown to disrupt adhesion (Kansas et al., 1993) and association with α-actinin (Pavalko et al., 1995), yet does not affect localization of L-selectin to the tips of microvilli projections (Pavalko et al., 1995). Calmodulin is known to regulate binding of proteins to cytoskeletal components through a "flip-flop" mechanism (Sobue et al., 1983; Hartwig et al., 1992). It is possible that calmodulin may modulate the interaction of L-selectin with α-actinin or with other molecules involved in signaling through L-selectin. Interestingly, deletion of the COOH-terminal 11 amino acid residues of the cytoplasmic domain does not affect L-selectin shedding (Chen et al., 1995). Based upon our observations, one might predict that truncation of the cytoplasmic tail would affect the association of calmodulin and thus increase the rate of L-selectin shedding. However, transfected cells expressing L-selectin already display a high level of spontaneous shedding of L-selectin, suggesting disregulation of the normal shedding process in transformed cells (Lasky et al., 1989; Kahn et al., 1994; Chen et al., 1995). Therefore, further truncation of the cytoplasmic tail may not increase the rate of shedding observed in transformed cells. Alternatively, we have mapped the CA21 MAb epitope to the COOH-terminal 8 amino acid residues of the L-selectin cytoplasmic tail (indeed, we have found that the terminal 8 amino acids can be engineered onto the COOH terminus of heterologous recombinant soluble proteins as a convenient epitope tag for the CA21 MAb). Since CA21 MAb does not inhibit calmodulin binding to L-selectin, it is possible that deletion of the COOH-terminal 11 amino acids may not be sufficient to abrogate the calmodulin influence on L-selectin expression. Resolution of this issue will require more extensive mapping studies.

Our data suggest a potentially novel role for calmodulin in regulating cell surface expression of an integral membrane protein through a protease-dependent mechanism. Recently it has become apparent that a number of other biologically important cell surface molecules, such as TNF-α, TGF-α, TNF receptors, IL-6 receptor, β-amyloid precursor protein, and angiotensin converting enzyme, are also regulated through activation-dependent proteolysis (Ehlers and Riordan, 1991; Arribas et al., 1996; Blobel, 1997; Mullberg et al., 1997). These proteins are also cleaved at a membrane-proximal site, and the cleavage is inhibited by hydroxamic

acid–based protease inhibitors. There has been considerable interest in identifying the protease(s) involved in the shedding of these diverse cell surface proteins. Recently a TNF-converting enzyme has been identified as a novel member of the adamalysin family of metalloproteases (Black et al., 1997; Moss et al., 1997). However, the mechanism by which these proteases are regulated is unknown. A CHO cell mutant defective in shedding of TGF-α has been selected and shown to be also defective in cleaving L-selectin, IL-6 receptor, and β-amyloid precursor protein, suggesting that there is some common component in the shedding process (Arribas et al., 1996). It will be of interest to determine if calmodulin plays a role in the regulation of these diverse cell surface proteins.

Experimental Procedures

Antibodies

The DREG-55 and DREG-200 MAbs, directed against the ectodomain of L-selectin; the JK924 serum, directed against the membrane-proximal cleavage region of L-selectin; the polyclonal antiserum JK564, directed against the entire cytoplasmic domain of L-selectin; and the CA21 MAb, directed against the COOH-terminal 8 amino acid residues of the L-selectin cytoplasmic domain, have been previously described (Kishimoto et al., 1990; Kahn et al., 1994). Phycoerythrin-conjugated anti-L-selectin (Leu-8), fluorescein isothiocyanate (FITC)–conjugated anti-CD45 (HLe-1), and labeled isotype control MAbs were purchased from Becton-Dickinson (Mountain View, CA). FITC-conjugated anti-Mac-1 MAb was purchased from Biosource International (Camarillo, CA). Anti-calmodulin MAb was purchased from Upstate Biotech (Lake Placid, NY).

Reagents

The KD-IX-73-4 hydroxamic acid–based protease inhibitor (Feehan et al., 1996) and the L-selectin cytoplasmic peptide (Kahn et al., 1994) have been previously described. Trifluoperazine, calmidazolium, and biotinylated calmodulin were purchased from Calbiochem (San Diego, CA). Purified calmodulin was purchased from Upstate Biotech (Lake Placid, NY).

Cells

Peripheral blood neutrophils were isolated from normal healthy volunteers by dextran sedimentation and ficoll-hypaque centrifugation, as previously described (Kahn et al., 1994). The L-selectin-transfected L1.2 pre-B cell line was a generous gift of Dr. E. Butcher, Stanford University. COS cells were transiently transfected with L-selectin cDNA by the DEAE-dextran method, as previously described (Migaki et al., 1995).

Mutagenesis

L-selectin mutants were generated using the Altered Sites oligonucleotide-directed mutagenesis system from Promega Biotech (Madison, WI), as previously described (Migaki et al., 1995). Mutant cDNAs were sequenced and checked for the presence of the engineered mutations and absence of any spontaneous mutations.

ELISA

Microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with either DREG-55 MAb or purified calmodulin. The plates were washed with PBS and nonspecific sites blocked with 2% BSA. Purified full-length L-selectin and recombinant soluble L-selectin were trapped on the DREG-55 plate and detected with either biotinylated DREG-200 MAb or biotinylated calmodulin. The biotinylated L-selectin cytoplasmic domain peptide was reacted with the calmodulin-coated plates. The biotinylated molecules were detected by incubation with a streptavidin-horseradish peroxidase conjugate, followed by development with the substrate 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS). Optical density was read at A_{405} nm.

Metabolic Cell Labeling and Immunoprecipitation

L-selectin transfectants were metabolically labeled with [^{35}S]methionine as previously described (Kahn et al., 1994; Migaki et al., 1995). Cell lysates and cell-free supernatants were immunoprecipitated as previously described (Kahn et al., 1994; Migaki et al., 1995). Immunoprecipitates were resolved on tricine-SDS polyacrylamide 10%–20% gradient gels (Novex, San Diego, CA). Gels were then fixed in 30% methanol, 10% acetic acid, 1% glycerol and treated with an autoradiography enhancer (Entensify A/B, DuPont-NEN, Boston, MA), dried in cellophane, and exposed to X-ray film (Kodak X-OMAT) at −70°C.

Western Blot Analysis

Cell lysates were immunoprecipitated with a Sepharose-bound CA21 MAb. Samples were resolved on a 10%–20% gradient SDS-PAGE gels (Novex) under nonreducing conditions and electrotransferred to PVDF membrane. The membrane was fixed with 0.2% glutaraldehyde, as previously described (van Eldik and Wolchok, 1984) and blocked in a 0.5% BSA in Tris-buffered saline (TBS) solution for at least 1 hr at room temperature. The blots were probed with CA21 MAb (0.5 μg/ml) or with anti-calmodulin MAb (1 μg/ml) followed by a horseradish peroxidase-sheep anti-mouse IgG conjugate (Amersham). The membranes were washed and visualized by addition of ECL chemiluminescent substrate and exposure to film, as per manufacturer's instructions (Amersham). In some experiments, alkaline phosphatase-conjugated goat anti-mouse IgG second-stage reagent (Promega) was used, and the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride.

FACS Analysis

Cells were treated with trifluoperazine or calmidazolium and/or fMLP as indicated and fixed gently with 0.5% paraformaldehyde to preserve antigen expression at specific time points, as previously described (Kishimoto et al., 1989). Cells were stained directly with 5 μl of phycoerythrin-conjugated anti-L-selectin MAb, FITC-conjugated anti-Mac-1 MAb, FITC-conjugated anti-CD45 MAb, or appropriate isotype control MAb in PBS containing 2% goat serum at 4°C for 30 min. Cells were then washed twice with goat serum/PBS, fixed in 1% paraformaldehyde/RPMI, and then analyzed by flow cytometry on a FACScan machine (Becton Dickinson, Mountain View, CA). In some experiments, cells were incubated with phycoerythrin-conjugated Leu-8 anti-L-selectin MAb and FITC-conjugated anti-Mac-1 or anti-CD45 MAb. Trifluoperazine and/or fMLP was added, and the cells were immediately analyzed in real time at 2 min intervals by flow cytometry. For each timepoint, 10,000 cells were analyzed. Both the cell fixation method and the real time analysis yielded similar results.

Neutrophil Rolling

Neutrophil rolling under hydrodynamic shear stress was performed with a parallel plate flow chamber (Glycotech, Gaithersburg, MD), as previously described (Walcheck et al., 1996a, 1996b). L-selectin ligands defined by the MECA-79 antigen were isolated from human tonsil on a MECA-79 MAb affinity column, as described (Lawrence et al., 1995; Finger et al., 1996). Experiments were performed at a wall shear stress of 1–2 dynes/cm^2. Neutrophils rolled on the MECA-79 antigen surfaces but not on uncoated surfaces. All interactions of neutrophils with MECA-79 antigen were eliminated by the addition of anti-L-selectin MAb (DREG-200). Neutrophils (5×10^5 cells/ml) were treated with KD-IX-73-4 hydroxamic acid–based metalloprotease inhibitor and/or trifluoperazine, as indicated, for 10 min at 37°C prior to infusion into the flow chamber. Control neutrophils were treated as above with an equivalent amount of DMSO used to solubilize the compounds. Within each experiment, the same field of the flow chamber was analyzed for both treated and untreated neutrophils. The order in which cells were analyzed—treated versus untreated groups—did not affect the results.

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