Real-time Analysis of Very Late Antigen-4 Affinity Modulation by Shear*

Received for publication, March 16, 2004, and in revised form, June 16, 2004 Published, JBC Papers in Press, June 28, 2004, DOI 10.1074/jbc.M402944200

Gordon J. Zwartz‡, Alexandre Chigaev‡, Denise C. Dwyer, Terry D. Foutz, Bruce S. Edwards, and Larry A. Sklar§

From the Department of Pathology and Cancer Research and Treatment Center, University of New Mexico, Albuquerque, New Mexico 87131

Shear promotes endothelial recruitment of leukocytes, cell activation, and transmigration. Mechanical stress on cells caused by shear can induce a rapid integrin conformational change and activation, followed by an increase in binding to the extracellular matrix. The molecular mechanism of increased avidity is unknown. We have shown previously that the affinity of the $\alpha_v\beta_3$ integrin, very late antigen-4 (VLA-4), measured with an LDV-containing small molecule, varies with cellular avidity, measured from cell disaggregation rates. In this study, we measured in real time affinity changes of VLA-4 in response to shear. The resulting affinity was comparable with the state mediated by receptor signaling and corresponded in time with intracellular Ca$^{2+}$ responses. Ca$^{2+}$ ionophores and N,N'-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-[2-(acetyloxy)ethoxy]-2-oxoethyl]], bis(acetyloxy)methyl]ester demonstrate that the affinity regulation of VLA-4 in the presence of shear was related to Ca$^{2+}$ signaling. Pertussis toxin treatment implicates G$i$ in an unknown pathway that connects shear, Ca$^{2+}$ elevation, VLA-4 affinity, and cell avidity.

Leukocytes are recruited to endothelial cells in a multistep process using selectin and integrin adhesion molecules (1, 2). These molecules allow a cell to tether, roll, adhere, and transmigrate along and across an endothelial layer. Selectin and some integrin molecules and their associated ligands mediate tethering and rolling interactions. Firm adhesion is mediated by vascular ligands of the immunoglobulin superfamily such as vascular cell adhesion molecule 1 (VCAM-1)$^1$ and their associated ligands mediate rolling. For example, intracellular signaling leads to conformational change and activation, followed by redistribution or clustering and to changes in the affinity of the receptor-ligand bonds (3–10).

Physiological shear can also regulate leukocyte traffic by stimulating mechanosensors on neutrophils, monocytes, lymphocytes, erythrocytes, and platelets (see Ref. 11 and references therein). Shear arises from bifurcating blood vessels or rapid changes in blood vessel diameters. Shear acting on leukocytes, bound to endothelial cells, produces mechanical stress on the cells or their receptors, regulating cell growth and proliferation, protein synthesis, gene expression, and blood cell recruitment (12, 13). Integrins such as $\alpha_v\beta_3$, $\alpha_v\beta_2$, $\alpha_v\beta_1$, and $\alpha_v\beta_2$ on endothelial cells can act as mechanosensors to changes in blood flow (13, 14) and trigger an intracellular signaling pathway involving focal adhesion kinase and mitogen-activated protein kinase cascades. How shear specifically induces blood cell adhesiveness or recruitment through mechanosensors is unknown. Indirect evidence shows that increased integrin binding to the extracellular matrix occurs when shear acts on cells or their mechanosensors to induce intracellular signaling. For example, intracellular signaling leads to conformational changes and activation of $\alpha_v\beta_3$ on endothelial cells and $\alpha_v\beta_2$ and $\alpha_v\beta_1$ integrins on monocytic cells (15, 16). Shear acting on endothelial cells affects the GTPase Rho signaling pathway and in monocytic cells induces inositol 1,4,5-trisphosphate-sensitive Ca$^{2+}$ release that affects cell adhesion avidity.

We have used an LDV-containing small molecule fluorescent probe to determine whether mechanical stress generated by shear can affect the affinity of VLA-4 by monitoring in real time the changes in VLA-4 affinity on live cells (17). We examined the contribution of intracellular signaling mechanisms to VLA-4 activation by shear. We found that VLA-4 affinity induced by shear was intermediate in affinity between the resting state and the Mn$^{2+}$-activated affinity state and similar to the physiologically activated receptor state generated using “inside-out” signaling (17). We found a temporal correlation between the intracellular Ca$^{2+}$ response and the higher VLA-4 affinity. We used Ca$^{2+}$ ionophores (A23187 and ionomycin) and BAPTA-AM to show that VLA-4 affinity regulation in response to shear was related to intracellular Ca$^{2+}$ signaling. Finally, we pretreated cells with pertussis toxin (PTX) to block G$i$ signaling and observed that VLA-4 activation was inhibited in the presence of shear. Our data suggest that shear regulates cell adhesion avidity by changing VLA-4 affinity and involves an incompletely characterized inside-out signaling pathway.

* This work was supported by National Institutes of Health Grants R01AI14175/EB002022 and HL56384 (to L. A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ These two authors contributed equally to this work.
§ To whom correspondence and reprint requests should be addressed: Dept. of Pathology and Cancer Research and Treatment Center, MSC 08-4630, 1 University of New Mexico, Albuquerque, NM 87131-0001. Tel: 505-272-6892; Fax: 505-272-6995; E-mail: lsklar@salud.unm.edu.
$^1$ The abbreviations used are: VCAM-1, vascular cell adhesion molecule 1; BAPTA-AM, N,N'$^2$-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-[2-(acetyloxy)ethoxy]-2-oxoethyl]], bis(acetyloxy)methyl]ester; FEP, fluorinated ethylene propylene; GPCR, G-protein-coupled receptor; LDV-containing small molecule, 4-((N'-2-methylphenyl)ureido)phenacyl-1-леucy1-1-aspartyl-1-valyl-1-prolyl-1-alanyl-1-alanyl-L-lysine; FITC, fluorescein isothiocyanate; LDV-FITC probe, 4-((N'-2-methylphenyl)ureido)phenacyl-1-леucy1-1-aspartyl-1-valyl-1-prolyl-1-alanyl-1-alanyl-L-lysine-FITC; MCF, mean channel fluorescence; PTX, pertussis toxin; VLA-4, very late antigen-4 ($\alpha_v\beta_3$ integrin); MOPS, 4-morpholinepropanesulfonic acid; BAPTA, N,N'$^2$-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-[2-(acetyloxy)ethoxy]-2-oxoethyl]]; AM, bis(acetyloxy)methyl]ester.

This paper is available on line at http://www.jbc.org

References

1 The abbreviations used are: VCAM-1, vascular cell adhesion molecule 1; MOPS, 4-morpholinepropanesulfonic acid; BAPTA, N,N'$^2$-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-[2-(acetyloxy)ethoxy]-2-oxoethyl]]; AM, bis(acetyloxy)methyl]ester.
EXPERIMENTAL PROCEDURES

Materials—The VLA-4-specific 4-[(N'-2-methylphenyl)ureido]-phenylacetyl-l-lysine (LDV containing a small molecule) and its FITC-labeled analog were synthesized at Commonwealth Biotechnologies, Inc. (Richmond, VA). Binding and dissociation of the LDV-FITC probe were described previously (17, 18). Intracellular Ca\(^{2+}\) was chelated using 5,5'-dimethyl-BAPTA-AM (acetoxymethyl ester) (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions. A23187 Ca\(^{2+}\) ionophore was purchased from Sigma and used at 1 \(\mu\)M concentration. Ionomycin was purchased from Calbiochem and used at 1 \(\mu\)M concentration. Fura Red AM and Flu-0 AM were purchased from Molecular Probes. FITC-conjugated monoclonal antibody, 44H6, against CD49d was purchased from Sero-tec (Raleigh, NC). All other reagents were from Sigma.

Cell Lines and Transfected Constructs—Human monoclastoid U937 cells were purchased from ATCC (Manassas, VA). Cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 10 mm HEPES, pH 7.4, 100 \(\mu\)g/ml ciprofloxacin, 2 \(\mu\)M l-glutamine, at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air. Site-directed mutants of formyl peptide receptor in the human monoclastoid line U937 constitutively expressing human VLA-4 integrin were prepared as described (19). High expressers were selected using the MoFlo Flow Cytometer (Cytomation, Inc., Fort Collins, CO). VLA-4 expression was measured with FITC-44H6 and quantified by comparison with a standard curve generated with Quantum Simply Cellular microspheres (Flow Cytometry Standards, San Juan, Puerto Rico) stained in parallel with the same monoclonal antibody. This produces an estimate of the total monoclonal antibody-binding sites/cell. Typically, we find 40,000–60,000 VLA-4 sites/U937 cell.

LDV-FITC Probe—The VLA-4 probe (20–22) was initially optimized from the ILDV binding sequence of the alternatively spliced connecting segment 1 of fibronectin. This sequence is homologous and isosteric with the QDID peptide found in the VCAM-1-binding site (23). The peptide sequence (Leu-Asp-Val-Pro-Ala-Ala-Lys-FITC) of the probe was based on structure-activity relationships of a potent VLA-4 binding inhibitor (compound 13 in Ref. 22). The specificity of the molecule for the peptide sequence (Leu-Asp-Val-Pro-Ala-Ala-Lys-FITC) of the probe was described (19). High expressors were selected using the MoFlo Flow Cytometer (Cytomation, Inc., Fort Collins, CO). VLA-4 expression was measured with FITC-44H6 and quantified by comparison with a standard curve generated with Quantum Simply Cellular microspheres (Flow Cytometry Standards, San Juan, Puerto Rico) stained in parallel with the same monoclonal antibody. This produces an estimate of the total monoclonal antibody-binding sites/cell. Typically, we find 40,000–60,000 VLA-4 sites/U937 cell.

Cell Preparation—U937 cells (10 \(\times\) 10\(^6\) cells/ml) for shear experiments were loaded with 6 \(\mu\)M Fura Red or 200 \(\mu\)M Flu o-4, for 30–60 min at 37 °C and gently mixed every 10 min. Then the cells were washed with complete RPMI and resuspended in phenol red-deficient RPMI (supplemented with 0.1% human serum albumin (Bayer Corp., Elkhart, IN), 1.5 \(\mu\)M Ca\(^{2+}\)). Cells were kept on ice after staining and washing. Typically, 5 min prior to each experiment, 4 \(\mu\)M LDV-FITC probe was added as a ligand to 1 \(\times\) 10\(^6\) cells/ml, and the sample was incubated in a 37 °C water bath. Cells were illuminated with 488-nm argon laser from a Becton-Dickinson FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA). Emission fluorescence was detected using a 585-nm band pass filter for Fura Red (FL2) and 530-nm band pass filter for Flu-0 (FL1). Fura Red fluorescence decreased when the indicator bound to free Ca\(^{2+}\). Changes in the affinity state of VLA-4 were monitored with the LDV-FITC probe. The probe was added 5 min prior to each experiment usually at 4 \(\mu\)M and incubated in a 37 °C water bath. Detailed analysis of real time binding and dissociation of the LDV-FITC probe was previously described in Refs. 17 and 18. In several experiments (where the extracellular Ca\(^{2+}\) concentration varied), Hepes buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, and 30 mM HEPES, pH 7.4) supplemented with 0.1% human serum albumin was used. Cell density was determined using a 22-Coulter counter (Coulter Corp., Miami, FL).

Intracellular Calcium Calibration—Molecular Probes calcium calibration kit 1 was used to generate a series of free calcium buffers that were used to obtain an intracellular cellular calcium calibration curve (Fig. 1). The kits contain two 50-m1 solutions, one solution containing 10 mM \(\text{Ca}^{2+}\) EGTA and the other the 10 mM EGTA. Both solutions contained 100 mM KCl, 30 mM MOPS, pH 7.2. Intermediate free calcium concentrations between 0 and 39 \(\mu\)M were obtained by cross-diluting the two buffers. Before adding U937 to each of the prepared buffers, the cells were stained with the intracellular calcium indicator Fura Red. Prior to each experiment, 1 \(\times\) 10\(^6\) U937 cells were added to 1 ml of a specific free calcium buffer. Then the solution was incubated for 5 min in a 37 °C water bath. A base line was established during the first 2 min of sampling with a FACScan to measure the resting state of the cells. Then 10 ng/ml of a calcium ionophore (A23187) was added and mixed gently, and sampling was resumed. Measurements of intracellular calcium were obtained when the Fura Red signal equilibrated. Fig. 1 shows that changes in the mean channel fluorescence (MCF) corresponded to logarithmic changes in the intracellular calcium levels. The intracellular Ca\(^{2+}\) calibration curve depended on Fura Red staining efficiency, viability of U937 cells, sensitivity of cells to external activation, and flow cytometer voltage and gain settings. The Fura Red MCF values for cellular resting states between 550 and 650 correspond to intracellular calcium concentrations between 100 and 10 nM. MCF values of ~400 after stimulation indicate an intracellular calcium concentration of ~1000 nM.

Creating Fluid Shear—Fluid shear was initially generated using a Fischer Scientific minivortexer (Fischer Scientific, Hampton, NH) set to 3200 rpm. The shear rate was estimated to be ~200–12,000 s\(^{-1}\), comparing the vortexed fluid motion inside a 12 (outer diameter) \(\times\) 75-mm tube with the fluid motion inside a Couette viscometer. The maximum (S\(_{\text{max}}\)) and minimum (S\(_{\text{min}}\)) wall shear rate for a given rotational velocity was approximated (24) as follows,

$$S_{\text{max}} = 2 \times R_1^2 \times \Omega (R_0^3 - R_1^3)$$

$$S_{\text{min}} = 2 \times R_1^2 \times \Omega (R_1^3 - R_0^3)$$

where \(R_1\) (ranging from ~0.535 to 0.25 cm) and \(R_0\) (0.55 cm) represent radii of the inner fluid and outer fluid surfaces, and \(\Omega\) is the angular speed of the inner cylinder.

Before being subjected to shear, U937 cells were incubated for 5 min in a 37 °C water bath. Each sample was gently mixed to resuspend cells, and a tube was attached to a flow cytometer. Data were acquired for 1–3 min to establish a base line for resting cells, and then each sample was removed from the flow cytometer to be exposed to shear for 5–30 s using a minivortexer. Samples were reattached to the flow cytometer, and data sampling was resumed.
A minivortexer generates turbulent fluid flow. To reduce this variability, we used a computer-driven syringe (Alitea, Bellevue, WA) to push samples through a 50-cm-long 0.03-inch (762-μm) inner diameter fluorinated ethylene propylene (FEP) tubing (Upchurch Scientific, Oak Harbor, WA) at flow rates of 33, 100, 200, and 400 μl/s. The capillary wall shear rates ($S_{wall}$) were calculated using the following,

$$S_{wall} = 4 \times \frac{Q}{\pi \times r^3} \quad \text{(Eq. 3)}$$

where $Q$ represents the flow rate, and $r$ is the tube radius (the corresponding wall shear rates were calculated to be 750, 2300, 4600, and 9200 s$^{-1}$, respectively), which was the maximal shear rate cells would experience (24). For deformable particulates, such as cells, there is a net radial hydrodynamic force moving particulates toward the flow axis (25), even at a low Reynolds number. Thus, not all cells flow along a capillary wall (maximal shear rate) or capillary axis (zero shear rate). Consequently, there was a range of shear experienced by flowing cells, and the maximal shear rate does not represent the shear experienced by all cells. For a simple approximation, we have assumed an average shear rate (between the maximum and minimum shear rate experienced by cells) for the four flow rates to be 375, 1150, 2300, and 4600 s$^{-1}$.

Fig. 3. Effect of capillary shear on the intracellular $\text{Ca}^{2+}$ and LDV-FITC probe binding to VLA-4 at different shear rates. A, intracellular calcium signaling in U937 cells was examined at 2300 s$^{-1}$ (200 μl/s; open circles), 1150 s$^{-1}$ (100 μl/s; open squares), and 375 s$^{-1}$ (33 μl/s; open triangles). Data were adjusted to the same base line and start time for flow into the FACScan. Resting and activated cell populations were selected from two time-gated regions denoted as Region B and A, respectively (see “Results”) (analysis of resting and activated cells is shown in C). B, same as A except LDV-FITC binding to VLA-4 data was examined (analysis of resting and activated cells is shown in D). C, normalized histogram of MCF calcium signaling data at 200 (2300 s$^{-1}$), 100 (1150 s$^{-1}$), and 33 (375 s$^{-1}$) μl/s obtained from A. The histograms were generated by selecting Region B in A (85–95 s; shear-activated) and Region A (12–22 s; resting state; in A). The resting state histogram was obtained by averaging histograms from Region A of 200 (2300 s$^{-1}$), 100 (1150 s$^{-1}$), and 33 (375 s$^{-1}$) μl/s data. The remaining histograms were obtained from Region B for the three flow conditions. All data were normalized to the largest value in the mean channel fluorescence distribution. D, same as C, except histograms of LDV-FITC probe binding to VLA-4 data were examined. E, percentage of U937 cells that were activated under shear conditions. A hyperbolic equation fit is shown for the LDV-FITC probe (filled circles) and $\text{Ca}^{2+}$ response (open circles). The percentage of activated cells was calculated using data presented in A and B.
shear. When that cycle was completed, the sample was aspirated into the same syringe. This cycle was repeated five times. After the fifth cycle, a computer-operated solenoid valve (Research, Caldwell, NJ), used to separate the shear FEP line from an FEP line leading to a FACScan, was switched to allow samples to be pushed toward a FACScan at 1 μs.

**Flow Cytometry and Data Analysis—**Flow cytometric analysis was done on a Becton-Dickinson FACScan flow cytometer (BD Biosciences). Data acquisition was performed using CellQuest (BD Biosciences). Data were analyzed offline using the Windows Multiple Document Interface Flow Cytometry Interface (Scirrps, La Jolla, CA). Time and fluorescence information were extracted from the data using FacsQuery software, developed by Bruce Edwards. Peak analysis and data fitting were done using PeakFit version 4.11 (Systat, Point Richmond, CA) and GraphPad Prism 4 (GraphPad, San Diego, CA), respectively.

A ligand dissociation analysis would not readily distinguish heterogeneity in the affinity of resting and activated receptors on a given cell as compared with heterogeneity in the distribution of receptors on activated and resting cells. However, the distribution of the amount of ligand bound would distinguish cells that had activated receptors from cells that did not. Thus, we have analyzed cell distributions before and after activation as shown in Fig. 3, regions A and B. The same principles were used for the analysis of ligand binding and Ca²⁺ response. For this analysis, a Gaussian curve was fitted to the mean channel fluorescence distribution obtained from region A, the resting state of cells. Region B was fitted with two Gaussian curves. One fitted the peak centroid and the full-width half maximum of Region A. The peak height was allowed to vary. This component represents resting cells. A second Gaussian curve was fitted to the remainder of the distribution in which the centroid and peak height were allowed to vary but full-width half maximum was fixed using the fit values obtained from Region A. The second curve represented activated cells. A simultaneous two-Gaussian fit to the mean channel fluorescence distribution obtained from Region B was done. The ratio of the total events under the two histograms was taken to estimate the fraction of cells activated under shear (resting—activated/activated).

**RESULTS**

**Fluid Forces Increase the Affinity of the αβ Integrin in Real Time—**Studies were conducted in a turbulent fluid flow environment using a Fischer Scientific minivortexer. To determine whether shear can affect the affinity of VLA-4, we used the LDV-FITC probe (17). Prior to applying shear, U937 cells (1 × 10⁶ cells/ml) were equilibrated with 4 nM probe. The concentration chosen for the experiments was below the dissociation constant (Kd of ~12 nM) for probe binding to resting VLA-4 and above the Kd for the physiologically activated receptor (Kd of ~1–2 nM) (17). Therefore, the transition from the low affinity to the high affinity receptor leads to an increased binding of the probe from ~25 to ~75% of receptor occupancy. Fig. 4 shows the rapid and transient increase in probe binding to sheared cells. The binding of the probe was detected after data acquisition was re-established, indicating that seconds were needed.
Fig. 7. VLA-4 activation and intracellular Ca\(^{2+}\) signaling in U937 cells treated with calcium ionophores (ionomycin and A23187) and BAPTA. 

A, effect of the extracellular buffer Ca\(^{2+}\) concentrations on VLA-4 activation in U937 cells. The inset shows the Ca\(^{2+}\) response (Fluo-4) to the addition of 1 μM ionomycin (solid line) and to a control for 1 mM extracellular Ca\(^{2+}\). The arrows indicate the time ionomycin was added.

B, LDV-FITC response for the U937 cells incubated without (filled circles) or with (open squares) 100 μM BAPTA (incubated 30 min prior to each experiment at 37 °C).

C, Ca\(^{2+}\) response (Fura Red) for cells that were incubated without (filled circles) or with (open squares) 100 μM BAPTA.
VLA-4 Affinity Modulation by Shear

Fig. 3.所示，静息细胞和剪切力分布图被归一化为与剪切力作用下的激活细胞数。图3显示了剪切力如何影响细胞数。那些被剪切力作用下的激活细胞数，通过使用Fura Red和VLA-4探针，在流式细胞仪上检测。所用的浓度为10^6/ml。结果量化在图3中，说明了剪切力对细胞数的影响。

**TABLE I**

| Cell treatment | k_{off} |
|----------------|---------|
| No treatment (1.5 mM Ca^{2+} + 1 mM Mg^{2+}) | 590–700° (resting) |
| N-Formyl-Met-Leu-Phe-Phe (1.5 mM Ca^{2+} + 1 mM Mg^{2+}) | 100–200° (activated) |
| 10 mM Ca^{2+} + 1 mM Mn^{2+} | 47 ± 5° |
| 1 mM Mn^{2+} | 7 ± 1° |
| Unvortexed (10 mM Ca^{2+} + 1 mM Mg^{2+}) | 684 ± 21 (resting) |
| Vortexed (10 mM Ca^{2+} + 1 mM Mg^{2+}) | 181 ± 6 (activated 76%) |

a Value of LDV-FITC probe dissociation rate from Chigaev et al. (17).

b Value of LDV-FITC probe dissociation rate from Chigaev et al. (18).

to induce cell activation. The binding reached a peak at 40–60 s after vortexing and decreased to the basal level after another 40–60 s. For comparison, we show the conformational state induced by 1 mM Mn^{2+} (K_v = 0.5 nM and occupancy = 90%) in the buffer containing 1 mM Mn^{2+} and 1 mM Ca^{2+} (18). Fig. 4 shows that Mn^{2+} increased probe binding above the level detected for shear.

Affinity Changes in a Controlled Fluid Force Environment—The range of shear rates was narrowed with computer-driven syringes and capillary tubes (see “Experimental Procedures”). Fig. 8, A and B, shows the kinetics of intracellular Ca^{2+} (determined using Fluo-4) and VLA-4 probe binding in response to the different levels of shear rates. The percentage of activated cells was calculated from Regions A and B in Fig. 3, and C, D, as described under “Experimental Procedures.” The resting state and shear histograms were normalized to the largest value in each of their distributions. Fig. 3, C and D, show how shear affected the number of activated cells. Those results are quantified in Fig. 3E, where the fraction of activated cells versus shear stress, fit to a hyperbolic equation, were comparable for both the LDV-FITC probe and intracellular Ca^{2+} responses.

**Simultaneous Observation of Integrin Activation and Intracellular Ca^{2+} Elevation in Response to Fluid Forces—**To follow VLA-4 affinity changes simultaneously with intracellular Ca^{2+} responses, the cells were stained with both Fura Red and the LDV-FITC probe. In several experiments, we used Fluo-4 to detect intracellular Ca^{2+} and VLA-4 activity in parallel. Fig. 5A shows Ca^{2+} and LDV-FITC binding responses after U937 cells were vortexed at 3200 rpm for 5, 15, and 30 s. The Fura Red fluorescent signal decreased as the intracellular Ca^{2+} concentration increased (the Fura Red axis in Fig. 5A is inverted). A transient and dose-dependent increase in intracellular Ca^{2+} was accompanied by an increase in the binding of the LDV-FITC probe. The kinetics of probe binding was similar, but the amplitude of signal was dependent on the duration of shear, reflecting differences in the number of activated cells (see “Affinity Changes in a Controlled Fluid Force Environment” and Fig. 3).

Fig. 5B shows a representative fluid flow experiment using a computer-driven syringe to produce a maximum wall shear rate of 9200 s^{-1} (average shear rate of 4600 s^{-1}; see “Creating Fluid Forces”). Resting cells were delivered to the flow cytometer at 1 μl/s for 1–2 min to obtain a base line. Then cells were sheared for ~30 s and delivered to the flow cytometer at 1 μl/s. Both the LDV-FITC probe binding and the intracellular Ca^{2+} signal increased and returned to their resting state at similar rates. The signal decay kinetics was significantly longer after capillary shear than for vortexing (compare Figs. 5B and 3, A and B, with Figs. 5A and 4). The kinetics of intracellular Ca^{2+} signaling as well as binding and dissociation of the LDV-FITC probe, observed simultaneously, vary in parallel in response to vortexing or capillary fluid flow.

Intracellular Ca^{2+} and Affinity Changes—To show the effect of intracellular Ca^{2+} on VLA-4 affinity, we activated cells through their G-protein-coupled receptors (GPCR), added Ca^{2+} ionophores (ionomycin and A23187), and chelated intracellular Ca^{2+} with BAPTA. It is known that VLA-4 can be activated through formyl peptide, CXCR2, CXCR4, and CCR3 receptors (17). Here, we took advantage of nucleotide receptors constitutively expressed on U937 cells (P_{2Y2} and P_{2Y6}).
that bind ATP to mediate a rapid and transient increase in intracellular Ca\(^{2+}\) (28). Fig. 6 shows that the addition of 1 \(\mu\)M ATP results in rapid increases in the Ca\(^{2+}\) signal with slower LDV-FITC probe binding amplitude similar to 30 s of vortexing. The binding of the LDV-FITC probe to the cells was limited by the rate of probe binding \(k_{\text{on}}\) \(= \sim 3\text{–}5 \times 10^7 \text{M}^{-1}\text{s}^{-1}\) and was somewhat slower than the actual VLA-4 activation rate (for comparison, see the probe binding kinetics in response to Mn\(^{2+}\)) (Fig. 4) (17, 18).

The dissociation of the LDV-FITC probe followed the slow decrease in the intracellular Ca\(^{2+}\) measured using Fura Red. This slow decay \((\sim 50\text{ s})\) reflected the kinetics of restoration of VLA-4 basal activity and was slower than probe dissociation from the resting state \(\sim 0.06 \text{ s}^{-1}\) (half-life of \(\sim 11\text{ s}\)) (17). Thus, the kinetics of VLA-4 activation on U937 cells coincides with the kinetics of intracellular Ca\(^{2+}\) signaling when the cell was activated through GPCR. The data were consistent with a resting Ca\(^{2+}\) concentration between 10 and 100 nm with elevation to \(-1000\text{ nm}\) following activation.

We used the Ca\(^{2+}\) ionophore ionomycin to increase the intracellular Ca\(^{2+}\) concentration. Ionomycin acts as a mobilizing agent across membranes and was used as a Ca\(^{2+}\)-mobilizing agent (29). After establishing a sample base line for 1 min, ionophores (1 \(\mu\)M ionomycin in Fig. 7A and 10 \(\mu\)g/ml A23187 in Fig. 7, B and C) were added. Cell activation was prevented during mixing by gently inverting the sample. Fig. 7A shows that ionomycin activated VLA-4 in the presence of 1 and 10 mM extracellular Ca\(^{2+}\), and the time course of the Ca\(^{2+}\) elevation was similar to the time course of VLA-4 activation. An increase in the extracellular Ca\(^{2+}\) concentration alone did not change the total binding of the LDV-FITC probe. Since both intracellular Ca\(^{2+}\) conditions led to similar total probe binding, it was likely that the two conditions had the same affinity state. However, the decay phase of the integrin activation was \(-3\) times longer in 10 mM Ca\(^{2+}\), suggesting that VLA-4 activation was strongly intracellular Ca\(^{2+}\)-dependent.

Intracellular Ca\(^{2+}\) was chelated by incubating cells with BAPTA. Then A23187 was added to elevate intracellular free Ca\(^{2+}\) (Fig. 7, B and C) and detected as a decrease in Fura Red fluorescence corresponding to an alteration from resting to elevated \((-1000\text{ nm})\) Ca\(^{2+}\) levels. The binding of the LDV-FITC probe increased at the same time (Fig. 7B). Bufferring intracellular Ca\(^{2+}\) with BAPTA allowed A23187 to induce a slow increase in the intracellular Ca\(^{2+}\) and LDV-FITC probe binding. Thus, the amount of the BAPTA \((100\text{ mM})\) loaded inside the cells was nearly sufficient to completely buffer Ca\(^{2+}\) influx. The slow increase in the binding of the LDV-FITC probe coincides with a slow increase in the intracellular free Ca\(^{2+}\).

**Effect of Fluid Forces on the LDV-FITC Probe Dissociation Rate**—We measured LDV-FITC dissociation rates of vortexed cells to characterize VLA-4 affinity under conditions where the duration of VLA-4 activation corresponds to the duration of the intracellular Ca\(^{2+}\) response \((-100\text{ s})\). Cells were incubated in 10 mM Ca\(^{2+}\), where the calcium signal lasts long enough to measure the LDV-FITC probe dissociation rate under shear (see Fig. 7A). The results are summarized in Table I and compared with a range of values found for other modes of VLA-4 activation. We found that the dissociation behavior after 10 s of vortexing required two exponential curves (fast and slow components) to fit the data. The fraction of the sites that appeared to remain in the resting state (fast component) was 24\%, whereas the remaining sites exhibited a dissociation rate 4 times slower (activated state). The rate was comparable with the physiological GPCR activation pathway or divalent cation conditions \((10 \text{ mM Ca}^{2+}\) and 1 mM Mn\(^{2+}\)). Intracellular pathways activated through extracellular stimuli \(\text{N-formyl-Met-Leu-Phe-Phe, interleukin-5, or IgE}\) all lead to VLA-4 of a similar affinity (17) and presumably in an extended conformational state of higher avidity. Our data suggest that intracellular signaling also occurs when cells are subjected to shear. Consequently, VLA-4 is activated to a similar affinity state as those generated from physiological stimuli.

**Chelation of Intracellular Ca\(^{2+}\) Prevents Integrin Affinity Changes in Response to Fluid Forces**—Fig. 8 shows the simultaneous LDV-FITC probe and intracellular Ca\(^{2+}\) response to shear in cells incubated with and without BAPTA. Cells that were treated with BAPTA do not respond to shear, whereas...
untreated cells do. Our results indicate that VLA-4 activation in response to shear was downstream of Ca\(^{2+}\) signaling and that an increase in intracellular Ca\(^{2+}\) was associated with activation of VLA-4.

**Pertussis Toxin Effect on Ca\(^{2+}\) Signaling and Integrin Affinity in Response to Fluid Force—Heterotrimeric G-proteins are part of a pathway that activates integrins (30). To determine whether heterotrimeric G-proteins were involved in the VLA-4 response to shear, U937 cells were treated with PTX. After establishing a base line of LDV-FITC probe binding, the sample was vortexed for 10 s, and sampling resumed (Fig. 9A). Treatment of the cells with PTX nearly abrogated the activation of VLA-4 by shear, suggesting that Go\(_{q}\)-related signaling can be an intermediate step in a mechanosensing pathway for VLA-4 activation. To test this hypothesis, we activated the same PTX-treated cells using P2Y receptors, constitutively expressed on activation. To test this hypothesis, we activated the same PTX-

**DISCUSSION**

**Fluid Forces, Intracellular Ca\(^{2+}\), and VLA-4 Affinity**—We have previously detected the real time regulation of VLA-4 affinity by divalent cations, physiological signaling, and reducing agents. Here we have shown that VLA-4 affinity was elevated in the presence of shear and that the effect was rapid and transient (Figs. 3–6, 8, and 9). A significant fraction of the cells, correlating with the receptors on them, responded to shear. The affinity of VLA-4 produced by this pathway was indistinguishable from the affinity produced by GPCR signaling.

The kinetics of intracellular Ca\(^{2+}\) signaling also corresponded to the time course of LDV-FITC binding to VLA-4 (Figs. 5 and 6) in the presence of shear. It was conceivable that the shorter vortex duration-induced responses (Fig. 4) as compared with the response to capillary fluid flow was due to shear produced during delivery through 0.03-inch internal diameter tubing that may preserve cells in an activated state for a longer period of time. In the absence of shear, Ca\(^{2+}\) ionophores (ionomycin and A23187) regulated VLA-4 affinity. Moreover, increased intracellular Ca\(^{2+}\) was always associated in time with VLA-4 activation in the presence of shear and that the effect was rapid and transient (Figs. 3–6, 8, and 9). A significant fraction of the cells, correlating with the receptors on them, responded to shear. The affinity of VLA-4 produced by this pathway was indistinguishable from the affinity produced by GPCR signaling.

To determine whether PTX-treated cells lose viability as represented by their capacity to respond through the Go\(_{q}\) pathway, we examined the Ca\(^{2+}\) dose-response curve for ATP (Fig. 10). A quantitative analysis was obtained by measuring the peak height of the Ca\(^{2+}\) response (measured with respect to a base line defined to be the time course before the addition of ATP) after the addition of ATP. The time courses of the ATP dose curve for cells treated with and without PTX were the same. Thus, the data indicate that cells treated with PTX were not adversely affected when compared with untreated cells.
examined the role of Go response to shear (Fig. 9A) by pre-treating cells with PTX. Because VLA-4 activation was associ- ated with intracellular Ca²⁺ signaling, we used ATP to initiate a Ca²⁺ response for cells treated with PTX. Those cells were activated through P₂Y receptors (Fig. 11 (V)), which were coupled to Go₄ (PTX-resistant (31–34)). Based on experiments with PTX, we observed that a functional Go/H9251 pathway and a mechanoreceptor (Fig. 11 (V)) were discussed. We tested whether blocking CD18 binding using antibodies (TS1/18; Endogen, Woburn, MA) to block the integrin activation (71), for intracellular Ca²⁺ mobilization (Fig. 11 (V)), or directly by fluid flow (Fig. 11 (V)).

Integrins are one of four classes of mechanosensors (43, 44) that include ion channels (45), G-protein receptors (46), and tyrosine kinase receptors (47). Each can be associated with intracellular Ca²⁺ signaling pathways (48–50). Connections among the classes are illustrated by G₁ and G₁₂/G₁₃ signaling pathways that are sufficient to activate α₁bβ₁ receptors on platelets (30). Whereas Go₄-mediated signaling is not essential for α₁bβ₁ activation but is for Ca²⁺ mobilization (Fig. 11 (V)), the overall mechanism connecting G protein receptors to integrin activation in platelets is unknown.

Fluid flow generated by a vortexer can affect suspended cells in several ways. Turbulent fluid motion produced stress on a cell membrane as a result of differential fluid velocities that can activate mechanosensors. In principle, fluid vortex motion can cause cells to collide in a nonbinding manner and activate receptors or the cell membrane. Alternatively, colliding U937 cells, potentially forming homotypic aggregates (doublets or triplets) through engagement of integrins and their ligands, would be subject to mechanical stress that would pull the aggregates apart and could initiate a cell signaling sequence and/or molecular extension. Cellular aggregates between VLA-4 and a U937 cellular ligand would be inhibited by the presence of LDV peptides binding specifically to VLA-4 (17).

VLA-4 Affinity Modulation by Shear

Our results were consistent with shear-induced mechanotransduction resulting in intracellular Ca²⁺ signaling and VLA-4 activation. The new VLA-4 affinity state observed under fluid flow was the same one induced by GPCR signaling, which was shown previously to increase the length of the VLA-4 molecule, to decrease the cellular avidity, and to decrease the ligand dissociation rate (17, 18). These VLA-4 structural and functional changes appear to parallel the global conformational rearrangement of the extracellular domains induced by ligands and divalent cation (74) and the switchblade model for the VLA-4 integrin based on electron microscopy, NMR, and epitope exposure data (75). Using fluorescence resonance energy transfer and the LDV-FITC probe (21), we found a striking correlation between the degree of VLA-4 extension and its affinity (76).

The prediction that force could increase adhesion bond strengths, catch bond (54), was verified by atomic force microscopy of P-selectin binding to P-selectin glycoprotein ligand-1 (77). We hypothesize that extension of an integrin could also be part of a braking system in leukocyte rolling (78) and that shear could play a role in the pathways shown in Fig. 11 (I, II, and III). We have obtained direct evidence for the first of these.²

REFERENCES

1. Springer, T. A. (1994) Cell 76, 301–314
2. Springer, T. A. (1995) Annu. Rev. Physiol. 57, 827–872
3. Carman, C. V., and Springer, T. A. (2003) Curr. Opin. Cell Biol. 15, 547–556
4. Chan, J. R., Hyduk, S. J., and Cybulsky, M. I. (2003) J. Immunol. Methods 273, 43–52
5. Faull, R. J., and Ginsberg, M. H. (1995) Stem Cells 13, 38–46
6. Grabovsky, V., Bleijs, D. A., Peled, A., Cinamon, G., Baleux, F., Arenasena-Seisedo, F., Lapidot, T., von Konsky, Y., Lobb, R. B., and Alon, R. (2000) J. Exp. Med. 192, 495–506
7. Lechoux, S., and Tedgui, A. (1998) Hypertension 32, 338–345
8. Chigaev, A., Blenc, A. M., Braaten, J. V., Kumaraswamy, N., Kepley, C. L., Andrews, R. P., Oliver, J. M., Edwards, B. S., Prossnitz, E. R., Larson, R. S., and Sklar, L. A. (2001) J. Biol. Chem. 276, 9327–9331
9. Trzina, E., del Pozo, M. A., Shattil, S. J., Chen, S., and Schwartz, M. A. (2001) EMBO J. 20, 4639–4647
10. Chigaev, A., Bleijs, D. A., Braaten, J. V., Kumaraswamy, N., Kepley, C. L., Andrews, R. P., Oliver, J. M., Edwards, B. S., Prossnitz, E. R., Larson, R. S., and Sklar, L. A. (2001) J. Biol. Chem. 276, 46760–46768
11. Chigaev, A., Blenc, A. M., Braaten, J. V., Kumaraswamy, N., Kepley, C. L., Andrews, R. P., Oliver, J. M., Edwards, B. S., Prossnitz, E. R., Larson, R. S., and Sklar, L. A. (2003) J. Biol. Chem. 278, 38174–38182
12. Kew, R. R., Peng, T., DiMartino, S. J., Madhavan, D., Weinman, S. J., Cheng, J. H., and Prossnitz, E. R. (1997) J. Biol. Chem. 272, 2399–2405
13. Chen, L. L., Whitty, A., Lobb, R. B., Prossnitz, E. R., Larson, R. S., and Sklar, L. A. (1998) J. Biol. Chem. 273, 329–337
14. Chen, L. L., Whitty, A., Lobb, R. B., Schmid-Schonbein, G. W., and Prossnitz, E. R. (1997) J. Exp. Med. 186, 37–46
15. Asahiya, A., Takemi, H., Kita, T., and Araki, H. (2003) J. Biol. Chem. 278, 9327–9331
16. Weiss, S. T., and Pollack, R. P. (1998) J. Cell. Biochem. 71, 554–561
17. Weber, C., Kitayama, J., and Springer, T. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10939–10944
18. Moazzen, P., DeLano, F. A., Zweifach, B. W., and Schmid-Schonbein, G. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5338–5343
19. Ingber, D. E. (2002) Circ. Res. 91, 877–887
20. Kew, R. R., Peng, T., DiMartino, S. J., Madhavan, D., Weinman, S. J., Cheng, J. H., and Prossnitz, E. R. (1997) J. Leukocyte Biol. 62, 329–337
21. Chen, L. L., Whitty, A., Lobb, R. B., Schmid-Schonbein, G. W., and Prossnitz, E. R. (1997) J. Biol. Chem. 272, 2399–2405
22. Lin, K., Ateq, H. S., Hsiung, S. H., Chong, L. T., Zimmerman, C. N., Castro, A. Lee, W. C., Hammond, C. E., Kulkunte, S., Chen, L. L., Pepinsky, R. B., Leon, D. R., Sprague, A. G., Abraham, W. M., Gill, A., Lobb, R. B., and

² G. J. Zwart, A. Chigaev, T. A. Feutz, B. S. Edwards, and L. A. Sklar, unpublished data.
23. Wang, J. H., Pepinsky, R. B., Stehle, T., Liu, J. H., Karpusas, M., Browning, B., and Osborn, I. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 5714–5718
24. Turitto, V. T., and Goldsmith, H. L. (1996) in *Vascular Medicine* (Veselko, E. M., Michel, C. C., and Geiger, S. R., eds) p. 141, Little, Brown, and Company, Boston
25. Chien, S., Usami, S., and Skalak, R. (1984) in *Handbook of Physiology* (Rennik, E. M., Michel, C. C., and Geiger, S. R., eds) p. 255, American Physiological Society, Bethesda, MD
26. Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J. M., Morelli, A., Torbello, M., Bolognesi, G., and Barcucci, O. R. (2000) *Blood* 95, 567–600
27. Jin, J., Dasari, V. R., Sistare, F. D., and Kunapuli, S. P. (2003) *J. Biol. Chem.* 278, 719–724
28. Kunapuli, S. P., and Daniel, J. L. (1993) *Nature* 361, 513–523
29. Wilson, B. S., Pfeiffer, J. R., Smith, A. J., Oliver, J. M., Oberdorf, J. A., and Butcher, E. C. (1991) *J. Exp. Med.* 174, 233–243
30. Zhou, Q., Wang, Y., and Butcher, E. C. (1999) *J. Immunol.* 162, 1652–1657
31. Kunapuli, S. P., and Daniel, J. L. (1993) *Biochem. J.* 306, 511–517
32. Berridge, M. J. (1993) *Nature* 361, 315–325
33. Warnock, R. A., Campbell, J. J., Dorf, M. E., Matsuzawa, A., McEvoy, L. M., and Butcher, E. C. (2000) *J. Exp. Med.* 191, 77–88
34. van Andrian, U. H., Chambers, J. D., McEvoy, L. M., Bargatz, R. F., Afors, K. E., and Butcher, E. C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 7528–7532
35. Martinez-Lemus, L. A., Emdad, L., Sklar, L. A., and Butcher, E. C. (2003) *J. Immunol.* 165, 7151–7158
36. Alon, R., Grabovsky, V., and Feigelson, S. (2003) *Science* 301, 297–311
37. Martin, E. H., Shah, J. K., and Butcher, E. C. (1999) *Science* 285, 719–724
38. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Science* 299, 224–228
39. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Nat. Immunol.* 4, 1151–1157
40. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Cell* 113, 1151–1161
41. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Science* 299, 224–228
42. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Cell* 113, 1151–1161
43. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Science* 299, 224–228
44. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Cell* 113, 1151–1161
45. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Science* 299, 224–228
46. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Cell* 113, 1151–1161
47. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Science* 299, 224–228
48. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Cell* 113, 1151–1161
49. Cinamon, G., Grabovsky, V., and Feigelson, S. (2003) *Science* 299, 224–228
