Integrin β1- and β3-mediated Endothelial Cell Migration Is Triggered Through Distinct Signaling Mechanisms

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Abstract. Human umbilical vein endothelial cell attachment, spreading and migration on collagen and vitronectin are mediated by integrins $\alpha_2\beta_1$ and $\alpha_v\beta_3$, respectively, and these events take place in the absence of cytokines, growth factors, or chemoattractants. Cell attachment and spreading on these ligands occur in the absence of extracellular calcium, as does migration on collagen. In contrast, vitronectin-mediated migration is absolutely dependent on the presence of extracellular calcium. Cell contact with immobilized vitronectin or anti-$\alpha_v\beta_3$ mAbs promotes a measurable rise in $[Ca^{2+}]_i$ which requires an extracellular calcium source, whereas collagen, or anti-$\alpha_2\beta_1$ mAbs fail to promote this signaling event. In fact, vitronectin-mediated migration

and the rise in intracellular calcium showed the same dose dependence on extracellular calcium. While vitronectin and collagen differ in their ability to induce a calcium influx both ligands or antibodies to their respective integrins promote an equivalent increase in intracellular pH consistent with activation of the Na/H antiporter an event independent of extracellular calcium. These results support two salient conclusions. Firstly, collagen and vitronectin, through their respective integrins, promote distinct intracellular signaling events. Secondly, the $\alpha_v\beta_3$ specific influx of calcium is not required for cell spreading yet appears to facilitate cellular migration on vitronectin.

CELLULAR migration plays a major role in development, wound healing, immune defense, and metastasis. To understand the molecular basis of cellular migration it is important to characterize the molecular event(s) underlying this biological process. It is clear that cell motility depends on the ability of cells to identify, attach and change shape in response to specific extracellular matrix components (Leavesley et al., 1992; Duband et al., 1991, 1990, 1986; Stoolman, 1989; Strauss et al., 1989; Tucker et al., 1988). Recent experimental evidence indicates that the integrin family of cell surface receptors not only mediate cellular adhesion and spreading but are directly involved in migration (Leavesley et al., 1992; Marks et al., 1991; Akiyama et al., 1989; Strauss et al., 1989; Tucker et al., 1988; McCarthy and Furcht, 1984). Integrins are known to promote highly stabilized adhesion to extracellular matrix (ECM)[1] proteins (Orlando and Cheresh, 1991), leading to reorganization of the actin–cytoskeleton, depending in part on the cytoplasmic tail of the integrin β subunit (Otey et al., 1990; Tapley et al., 1989; Horowitz, 1986). Thus, integrins provide a functional linkage between the extracellular environment and the intracellular compartment. (Tamkun et al., 1986; Buck and Horowitz, 1987).

The nature of the integrin-induced transmembrane signal(s) leading to the locomotory response have yet to be defined, although some early integrin-stimulated second messengers have been described. For example, alterations in cytosolic free calcium ($[Ca^{2+}]_i$), and intracellular pH (pH_i) are observed during interactions with ECM components (Jaconi et al., 1991; Ng-Sikorski et al., 1991; Ingber et al., 1990; Schwartz et al., 1991; Altieri et al., 1990). Recent studies have shown that endothelial cell adhesion to matrix proteins triggers a detectable influx of extracellular calcium via voltage-independent calcium channels (Schwartz, 1993). Alterations in $[Ca^{2+}]_i$ directly affect the function of several actin-binding proteins, altering the organization of the actin network (Stossel, 1989). Various Ca^{2+}-sensitive kinases and phosphatases are associated with, and/or act upon the cytoskeleton (Gaudry et al., 1992; Hendey and Maxfield, 1992; Wilson et al., 1991). Thus $[Ca^{2+}]_i$ is a candidate integrin second messenger with potential to directly or indirectly regulate cytoskeletal structure and consequently cell spreading and motility.

In the current study we have examined the potential role of calcium on endothelial cell migration. These studies were performed in the absence of any growth factors, cytokines, chemoattractants, or serum, allowing us to examine cellular migration due to individual ECM components. We demonstrate that endothelial cell attachment and spreading on vitronectin or collagen leads to an elevation of pH_i which is independent of extracellular calcium. Cell contact with

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1. *Abbreviations used in this paper:* Ac LDL, acetylated low-density lipoprotein; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cells; pH_i, intracellular pH.

vitronectin via integrin $\alpha_v\beta_3$ induces a specific rise in $[Ca^{2+}]_i$, correlating with a requirement for calcium during $\alpha_v\beta_3$-mediated cell migration. In contrast, cellular interaction with collagen via $\alpha_2\beta_1$, fails to induce a change in $[Ca^{2+}]_i$ and, correspondingly, migration on collagen is calcium independent.

Materials and Methods

Cells and Cell Culture

Primary human umbilical vein endothelial cells (HUVEC; Jaffe et al., 1973) were maintained in M199, 20% FBS, 10 mM Hepes, 9 mg/ml heparin, 3 μg/ml endothelial cell growth supplement (H-Neurext™; Upstate Biotechnology Inc., Lake Placid, NY), 50 μg/ml gentamicin.

Antibodies

Integrin-specific mAbs LM609 (anti-$\alpha_v\beta_3$; Cheresh and Spiro, 1987), LM142 (anti-α_v; Cheresh and Harper, 1987), P4C10 (anti-β_1; Carter et al., 1990), P3G2 (anti-$\alpha_v\beta_5$; Wayner et al., 1991), and W6/32 (anti-HLA class I: Brodsky and Parham, 1982) were affinity purified from ascites on protein A-Sepharose (MAPs II Kit, BioRad Laboratories, Richmond, CA) as described previously (Leavesley et al., 1992). mAb 6F1 (anti-$\alpha_2\beta_1$; Coller et al., 1989) was kindly provided by B. Coller (State University of New York, Stony Brook, NY), and 12F1 (anti-α_2; Pischel et al., 1987) by V. Woods (University of California, San Diego, CA). All antibodies are murine and of IgG1 isotype except W6/32 which is an IgG2a.

Adhesive Ligands

Vitronectin was prepared from out-dated human plasma as described by Yatohgo et al. (1988). Collagen (rat tail) type I was purchased from Collaborative Research (New Bedford, MA). BSA was purchased from Sigma Immunochemicals (St Louis, MO), acetylated low-density lipoprotein (Ac LDL) from Biomedical Technologies (Stoughton, MA).

Adhesion Assay

Cell adhesion assays were performed as previously described (Leavesley et al., 1992; Wayner et al., 1991). In brief, sterile, untreated, bacteriological-grade polystyrene 48-well cluster plates (Costar Corp., Cambridge, MA) were coated for 1 h at room temperature with adhesive ligands (10 μg/ml) diluted in PBS and subsequently blocked with heat-denatured 1% BSA in PBS, pH 7.4. HUVECs were harvested using trypsin/EDTA, washed, resuspended in M199, 10 mM Hepes, 0.5% BSA, and radiolabeled with ^{51}Cr (Amersham Corp., Arlington Heights, IL) for 30 min at 37°C. Labeled cells were washed three times and resuspended in 20 mM Hepes, pH 7.45, 150 mM KCl, 5 mM glucose, 0.5% BSA (Adhesion Buffer), and 10^5 cells in 0.5 ml placed into appropriate wells. Purified mAbs specific for various integrins (25 μg/ml), or 5 mM EGTA were included where indicated. Cells were permitted to adhere for 45 min at 37°C after which nonadherent cells were removed with washing. Attached cells were lysed with 0.25% SDS, 0.1 M NaOH and enumerated by gamma counting. Cell spreading was evaluated in this procedure under phase contrast optics and random fields were photographed.

Cell Migration Assay

Cell migration assays were performed using 6.5 mm, 8.0 μm pore size Transwells (Costar Corp.) as previously described (Leavesley et al., 1992) with some modifications. Soluble ECM ligands (5 μg/ml) diluted in 20 mM Hepes, pH 7.45, 150 mM NaCl, 1.8 mM CaCl$_2$, 1.8 mM MgCl$_2$, 5 mM KCl, 5 mM glucose (HBS), were placed into a 24-well plate containing Transwell inserts and allowed to stand at 37°C for ~30 min before placement of cells into the insert. Sub-confluent, early passage (1-6) HUVEC cultures were harvested with trypsin/EDTA, washed, and resuspended to 10^6 cells/ml in adhesion buffer. Where indicated, cells were preincubated for 5-20 min in the presence of various mAbs (25 μg/ml) or 5 mM EGTA. Cell migration was measured after 4 h of incubation at 37°C. All nonmigrant cells were removed from the upper face of the Transwell membrane with a cotton swab and migrant cells, those attached to the lower face, were fixed and stained with 0.1% crystal violet in 0.1 M borate, pH 9.0, 2% etha-

nol. Stained cells were subsequently extracted with 10% acetic acid, absorbance at 600 nm determined, and migration enumerated from a standard calibration curve. Random migration was determined in the absence of a chemoattractant gradient, i.e., matrix proteins were included in both upper and lower chambers. Nonspecific migration was determined using BSA as a ligand. In a typical experiment after 4 h of migration between 40 and 60% of the cells migrate to the lower surface of the membrane. In control experiment in the presence of BSA <1% of the cells migrate.

Intracellular Calcium and pH Measurements

For measurements of intracellular calcium, cells were incubated with 2-4 μM fura-2-AM (Molecular Probes, Inc., Eugene, OR) for 20-40 min, rinsed and plated. A well was formed by cutting a circular hole in a 35-mm plastic petri dish and attaching a glass coverslip to the outside of the dish with silicone vacuum grease. Cells were plated in the well on the stage of a Nikon diaphot microscope, equipped with the Photoscan 2 system for dual excitation microscopy. Light from the xenon-arc source passes through a variable diaphragm and is directed by a chopper, alternating at 100 Hz, through either of two band pass filters centered on 340 and 380 nm with 20 nm bandwidths (Omega Optical Inc., Brattleboro, VT). Monochromatic light is directed via fiber optic cables through a 485-nM dichroic mirror and a 20× quartz 0.75 N.A. Nikon Fluor objective onto the sample. Emitted light passes through a second diaphragm, selecting only a small portion of the field and directing it through a 510 nm ($\pm$20 nm) band pass filter into the photon counter. For most of the experiments reported here, the diaphragm was set to measure emitted light from a single cell in the center of the field. The system is controlled with an NEC 286 computer. Background measurements were made from nearby cell-free areas, and fluorescence intensities corrected accordingly. The 380/340 nm ratio is calculated and $[Ca^{2+}]_i$ estimated from a calibration standard curve determined using fura-2 dissolved in pH 7.2 20 mM Hepes, 100 mM KCl, 10 mM EGTA, and known CaCl$_2$ concentrations.

Temperature and gas were controlled by a modified version of the apparatus previously described (Schwartz et al., 1989). 35-mm plastic dishes were placed inside a chamber heated from a recirculating water bath. Air from an aquarium air pump was mixed with CO$_2$ to 7.5%, humidified by bubbling through warm deionized water, passed through a stainless steel tube surrounded by a heating element, and directed into the chamber to blow across the surface of the medium. The heating element was controlled by a (Yellow Springs, OH) temperature controller (model 72; YSI, Yellow Spring, OH) and monitored by a YSI511 temperature probe placed directly in the medium. This system provides a constant 37 $\pm$ <0.5°C, without overshoots.

Coverslips were coated with 40 μg/ml collagen or vitronectin in PBS for 1 h and blocked with 1% heat denatured BSA. Cells were harvested with EDTA/PBS, transferred to DME, held in suspension for 2 h, loaded with fura-2 AM, and plated. At each time point, ~15 individual cells were measured for 6 s in quick succession.

Measurement of pH$_i$ in cells loaded with 2 mM BCECF-AM (Molecular Probes Inc.) used the same dual excitation protocol using 440 and 490 nm (20-nm bandwidth) excitation filters with a 530 nm (20-nm bandwidths) emission filter and 515-nm dichroic mirror (Chroma Technologies Corp., Brattleboro, VT). pH$_i$ measurements were calibrated using high potassium buffer and nigericin as described previously (Vairo et al., 1990; Ingber et al., 1990). Small areas of 35-mm untreated, bacteriological-grade polystyrene dishes were coated for 1 h with 20 μl droplets of collagen, vitronectin, or Ac LDL (100 μg/ml). In antibody studies, small areas were coated for 1 h with goat anti-mouse IgG (50 μg/ml), blocked with 1% heat-denatured BSA for 20 min and then incubated with 20 μg/ml anti-integrin or anti-Human Class I Histocompatibility Antigens (HLA) IgG for 1 h. Cells were plated in dishes containing several small areas coated with different adhesive proteins or antibodies. After 1-2 h of attachment, pH$_i$ was measured in ~15 individual cells for 6 s each in quick succession.

Results

Adhesive Proteins Stimulate Endothelial Cell Migration

HUVECs migrate in the absence of serum, growth factors, or exogenous chemoattractants through a microporous membrane toward collagen or vitronectin in a time-dependent
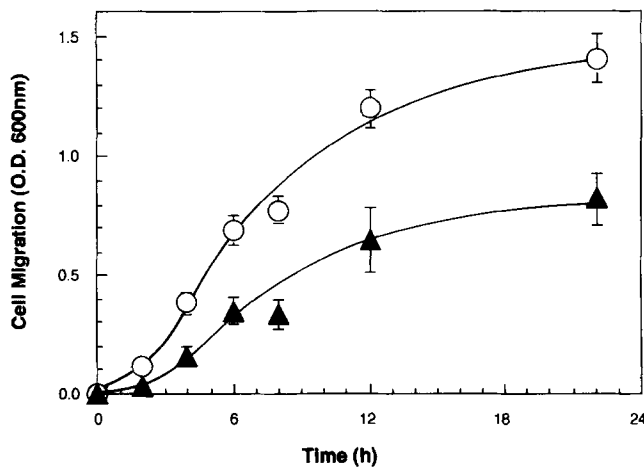
Figure 1. Endothelial cell migration to adhesive proteins. HUVECs (100,000) suspended in 1% BSA, HBS, were placed into Transwells and incubated at 37°C for the indicated periods. Soluble collagen (O) or vitronectin (▲) at 5 μg/ml in HBS (see Materials and Methods) was included in the lower "migration" chamber. Transwells were removed, cells on the upper surface removed, and migrant cells adhering to the lower face of the Transwell membrane, fixed, stained, and enumerated from dye uptake. Nonspecific migration (toward BSA) has been subtracted. Each point represents the mean ± SD of quadruplicates.

manner (Fig. 1). In the presence of a defined buffered salt solution containing 2 mM calcium and 2 mM magnesium, cell migration towards both ligands is apparently linear for several hours (Fig. 1). Endothelial cell migration measured on these ligands occurs in a concentration-dependent manner, becoming maximal at 5–10 μg/ml for both collagen and vitronectin (Fig. 2). After 4 h of incubation at 37°C, when
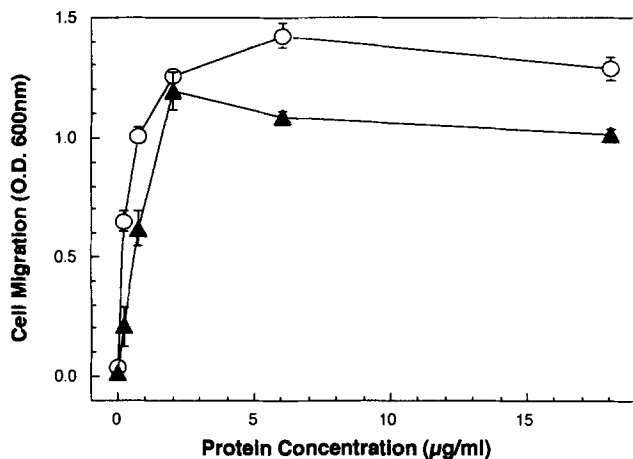
Figure 2. Dose dependency of ECM-induced endothelial cell migration. Collagen (O) or vitronectin (▲) at various concentrations (see Materials and Methods) was placed into the lower migration chamber of a Transwell plate. HUVECs (100,000) were placed into the Transwell insert and allowed to migrate for 4 h at 37°C. Transwells were removed, cells on the upper membrane surface removed and migrant cells fixed, stained, and enumerated from dye uptake as described in Materials and Methods. Nonspecific migration has been subtracted. Each point represents the mean ± SD of quadruplicate inserts.

migration is still first order, 40–60% of the loaded cell population can be detected attached and spread on the lower face of the Transwell membrane.

Integrins $\alpha_v\beta_3$ and $\alpha_2\beta_1$ Mediate Cell Migration to Vitronectin and Collagen, Respectively

A variety of cells are known to use β_3 and β_1 integrins to attach and spread on vitronectin and collagen, respectively (Hynes, 1992; Cheresh, 1992; Ruoslahti, 1991; Albelda and Buck, 1990). Thus, to determine whether HUVEC migration also depends on these integrins, cell migration assays were performed in the presence of specific anti-integrin IgG. As shown in Fig. 3, mAb 6F1, directed to a functional epitope on integrin $\alpha_2\beta_1$ (Coller et al., 1989), abolished migration on collagen but had no effect on vitronectin. Similarly, mAb P4C10, directed to β_1, also blocked cell migration on collagen but failed to affect vitronectin-mediated migration. In contrast, mAb LM609, directed to integrin $\alpha_v\beta_3$, significantly inhibited (73%) migration on vitronectin and had no effect on collagen-dependent migration. These data demonstrate that endothelial cell migration in response to vitronectin and collagen are primarily mediated by integrins $\alpha_v\beta_3$ and $\alpha_2\beta_1$, respectively.

Role of Calcium in the Endothelial Cell Migration Response

To determine the potential role of calcium in endothelial cell migration, cells were allowed to migrate towards collagen or vitronectin in the presence or absence of 5 mM EGTA. Cell migration to both adhesive proteins was readily detected in the presence of 2 mM calcium and 2 mM magnesium (Fig. 1). However, when EGTA was included to specifically chelate calcium, vitronectin-mediated migration was completely abolished while migration to collagen remained measurable (Fig. 4). In parallel experiments, cell attachment and spreading was equivalent on both ligands whether or not calcium
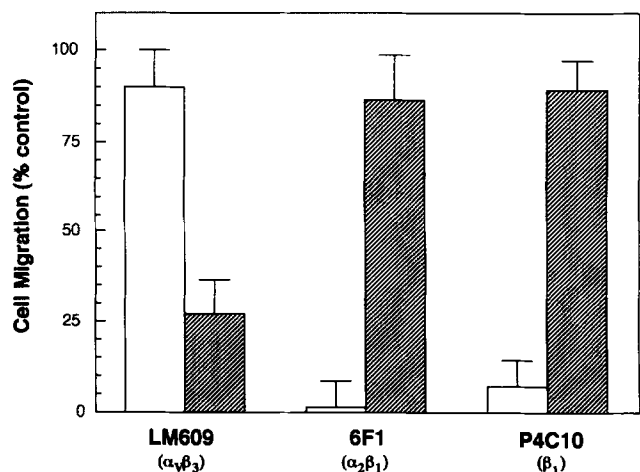
Figure 3. Antibody specific inhibition of endothelial cell attachment and migration on adhesive proteins. HUVEC migration towards collagen (□) and vitronectin (▨) was determined in the presence of indicated antibodies diluted in HBS (25 μg/ml) after 4 h incubation at 37°C as described in Materials and Methods. Each bar represents the mean percentage migration relative to an antibody free control ± SD of quadruplicate Transwells.
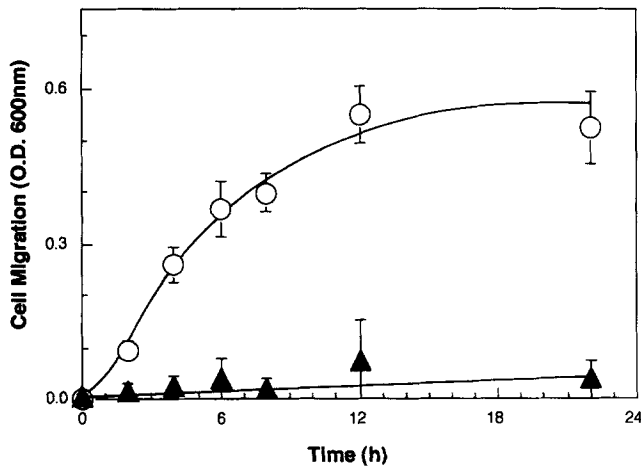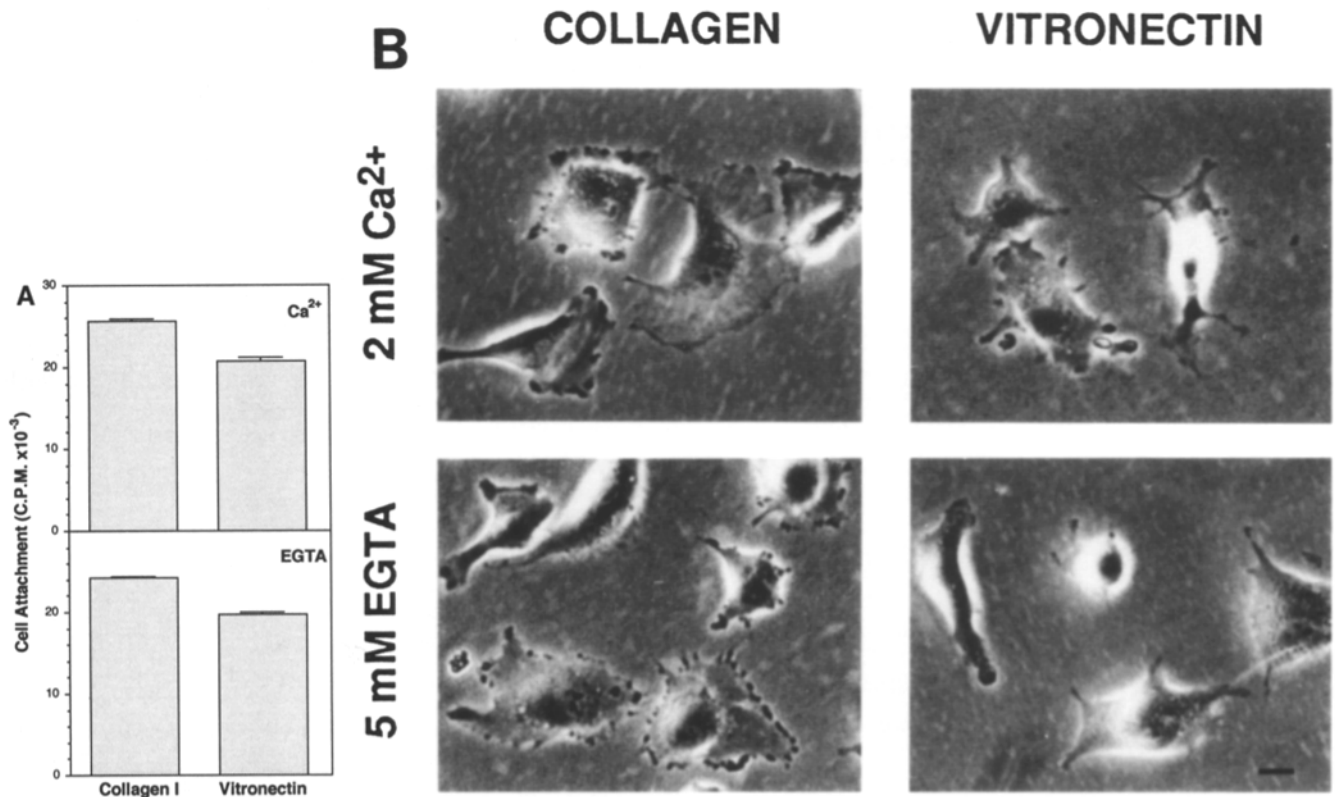
Figure 4. Calcium-free endothelial cell migration to adhesive proteins. HUVECs (100,000) suspended in HBS with 1% BSA containing 5 mM EGTA, were placed into Transwells and incubated at 37°C. Soluble collagen (O) or vitronectin (▲) at 5 μg/ml in 5 mM EGTA, HBS (see Materials and Methods) was included in the lower migration chamber. After the indicated periods Transwell inserts were removed, cells on the upper membrane surface removed and migrant cells adhering to the lower membrane face, fixed, stained, and enumerated from dye uptake. Nonspecific migration has been subtracted. Each point represents the mean ± SD of quadruplicates.

was present (Fig. 5, *A* and *B*). These data demonstrate that cell adhesion and spreading on collagen and vitronectin occur in a calcium-independent manner. In contrast, vitronectin-mediated migration requires calcium even though migration on collagen does not. Cell migration on vitronectin could be fully restored in EGTA-treated cells by returning cells into a calcium-containing buffer, indicating that EGTA treatment was reversible and nontoxic (not shown).

Intracellular Calcium

To investigate whether the requirement for extracellular calcium during vitronectin-dependent cell migration was related to this intracellular signaling event, cells were loaded with the calcium-sensitive dye fura-2 and allowed to contact vitronectin- or collagen-coated substrata. As shown in Fig. 6 vitronectin promoted a steady and significant increase in $[Ca^{2+}]_i$ representing a rise of ~40 nM in 45–60 min. In contrast, cells attached to collagen showed no significant increase in $[Ca^{2+}]_i$ over the course of the assay. Importantly, cell spreading on both of these ligands was complete within the duration (30 min) of this experiment and took place in the absence of calcium (e.g., in the presence of 5 mM EGTA, see Fig. 5 *B*). Collagen failed to promote a rise in intracellular calcium even after 4 h of adhesion (not shown).

To establish whether $\alpha_v\beta_3$ ligation was sufficient to pro-

Figure 5. Attachment and spreading of endothelial cells on adhesive proteins under calcium free conditions. (*A*)[51]Cr-radiolabeled HUVECs suspended in adhesion buffer ± EGTA (5 mM) were allowed to attach and spread for 45 min at 37°C in 48-well plates precoated with adhesive ligands (10 μg/ml) and blocked with heat–denatured BSA (1%) (see Materials and Methods). Nonattached cells were removed with three washes (250 μl) of adhesion buffer and attached cells lysed and released radioactivity determined in a gamma counter. Each point represents the mean ± SD of six replicates. (*B*) Cells were allowed to attach as above and adherent cells photographed at 200× using phase–contrast optics with Kodak T-MAX 100 ASA Film (Eastman Kodak Co., Rochester, NY). Bar, 10 μm.
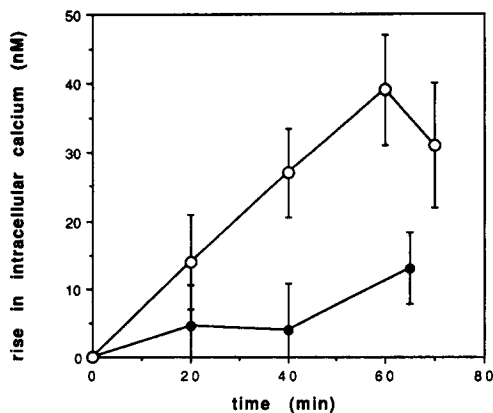
Figure 6. Time course of $[Ca^{2+}]_i$ on vitronectin or collagen. HUVECs loaded with fura-2 were plated on glass coverslips coated with collagen (●) or vitronectin (○) as described in Materials and Methods. At the indicated times, $[Ca^{2+}]_i$ was determined for ≥15 cells as described in Materials and Methods. The mean increase in $[Ca^{2+}]_i$ ± SD, relative to time zero is shown.

mote the rise in $[Ca^{2+}]_i$, cells were allowed to attach and spread on immobilized matrix proteins or anti-integrin IgG. As shown in Fig. 7 and Table I, both vitronectin and mAbs to $\alpha_v\beta_3$ promoted equivalent elevations in $[Ca^{2+}]_i$, whereas collagen or mAbs to $\alpha_2\beta_1$ did not, giving responses similar to the control proteins Ac LDL and anti-HLA.

To determine if the $\alpha_v\beta_3$-specific rise in $[Ca^{2+}]_i$ was related to the EGTA-sensitive, vitronectin-specific HUVEC migration response (Fig. 4), EGTA was added to cells 1 h after plating and measurements of $[Ca^{2+}]_i$ repeated. As shown in Fig. 7, chelation of extracellular calcium reversed the increase in $[Ca^{2+}]_i$ induced by vitronectin or anti-α_v IgG but had no affect on baseline $[Ca^{2+}]_i$. These results demonstrate that both the $\alpha_v\beta_3$-dependent calcium response and
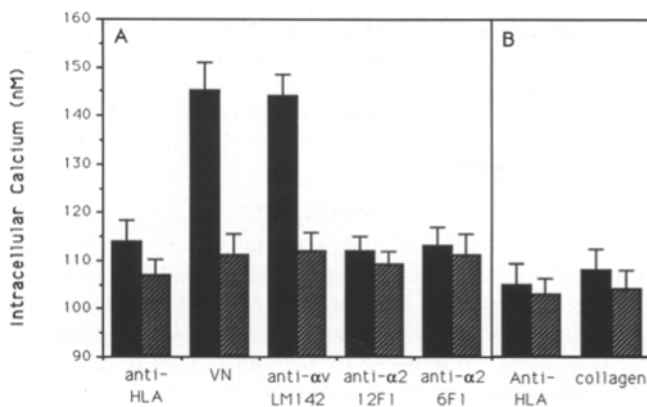
Figure 7. Effect of adhesive ligands and anti-integrin antibodies on endothelial cell $[Ca^{2+}]_i$. (A) HUVECs loaded with fura-2 were plated on coverslips coated with vitronectin or antibodies. After incubation for 1 h at 37°C $[Ca^{2+}]_i$ was measured as described in Materials and Methods, 10 mM EGTA added after which $[Ca_2^+]_i$ was measured again. Each bar represents the mean ± SD of at least 15 cells. (B) Cells were allowed to attach to collagen or anti-HLA as above and intracellular calcium was measured in the presence or absence of EGTA. (■) Control; (▨) 10 mM EGTA.

Table I. Effect of Adhesive Ligands or Anti-Integrin Antibodies on Endothelial Cell Intracellular Ca²⁺

Ligand	$\Delta[Ca^{2+}]_i$ (nM) mean ± SD	Antibody (antigen)	$\Delta[Ca^{2+}]_i$ (nM) mean ± SD
Vitronectin	25 ± 4	LM142 (α_v)	27 ± 3
Collagen	1 ± 4	LM609 ($\alpha_v\beta_3$)	25 ± 1
BSA	2 ± 4	12F1 (α_2)	3 ± 2
AcLDL	2 ± 4	6F1 ($\alpha_2\beta_1$)	4 ± 1
		W6/32 (HLA)	2 ± 1

HUVECs loaded with fura-2 were plated on glass coverslips precoated with adhesive ligands as described in Materials and Methods. The level of $[Ca^{2+}]_i$ in attached cells was determined after 45–60 min and mean ± SE from seven experiments (≥15 cells per experiment) calculated. $\Delta[Ca^{2+}]_i$ is the total level of $[Ca^{2+}]_i$ less the level determined in cells pretreated with 10 mM EGTA.

vitronectin-mediated migration are sensitive to removal of extracellular calcium, suggesting these events are related.

While ligand-specific changes in $[Ca^{2+}]_i$ were detected, ligation of either $\alpha_v\beta_3$ or $\alpha_2\beta_1$ stimulated equivalent increases in pH_i (Fig. 8), consistent with activation of the Na/H antiporter. As shown in Fig. 8, cell attachment to collagen, vitronectin, anti-$\alpha_2\beta_1$ IgG, or anti-$\alpha_v\beta_3$ IgG promoted comparable rises in pH_i. These data demonstrate that cell attachment to collagen or vitronectin via these integrins does not differ with respect to activation of the Na/H antiporter. We show that EGTA does not inhibit the vitronectin-induced rise in pH_i (Fig. 8 B), indicating $\alpha_v\beta_3$ activation of the Na/H antiporter is independent of extracellular and intracellular calcium, and further demonstrating that perturbations of $[Ca^{2+}]_i$ do not affect the ability of $\alpha_v\beta_3$ to promote transmembrane signals.

To determine whether the extracellular calcium level correlated with its ability to promote vitronectin migration and a concomitant rise in intracellular calcium experiments were performed with defined levels of extracellular calcium. In this case EGTA was added at varying concentrations to migration buffer containing 1.8 mM calcium and magnesium and cell migration and intracellular calcium was measured. As shown in Fig. 9 the dose–response curves for vitronectin-dependent migration and intracellular calcium were identically dose-dependent. The extracellular calcium concentration required for half-maximal migration and intracellular calcium was ~1 mM. In contrast, collagen migration was not effected by changes in the extracellular or intracellular calcium levels (Fig. 9).
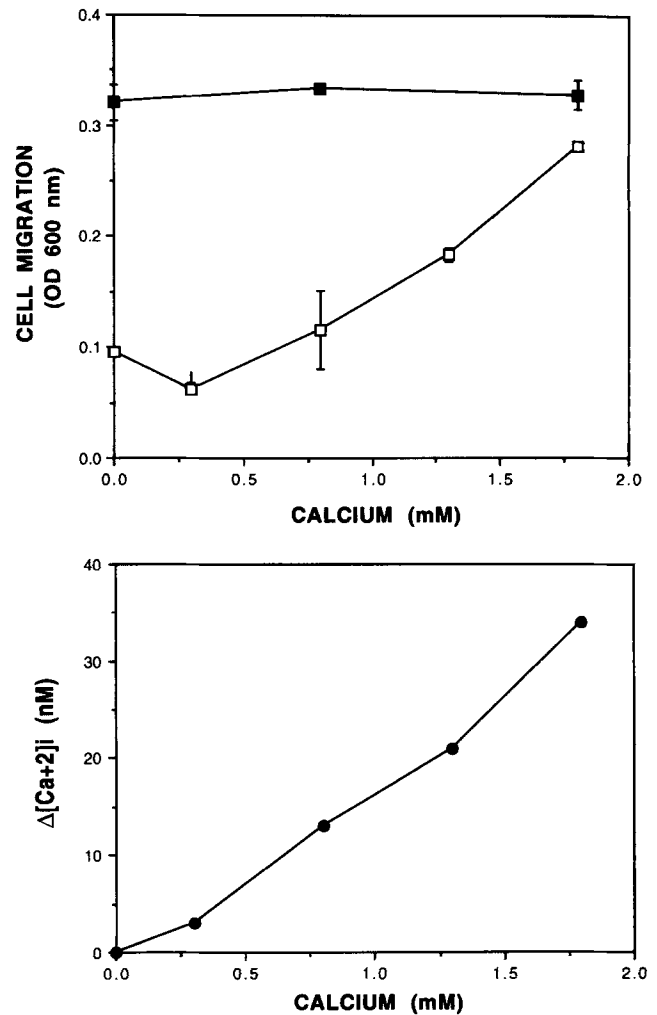
Discussion

Endothelial cell migration is important during development, wound healing and angiogenesis. The capacity of endothelial cells to recognize and migrate in response to the ECM depends on one or more members of the integrin family of cell adhesion receptors. The molecular basis of integrin-mediated cell migration is poorly understood but probably depends on signal transduction events triggered by integrins during the initial phases of cell adhesion. Little is known of the second messengers potentiating cell migration or, for that matter, if common or distinct signals trigger cell spreading versus cell migration responses. For example, cells often spread upon contact with an adhesive ligand but do not necessarily migrate, even though cell spreading is thought to be a prerequi-
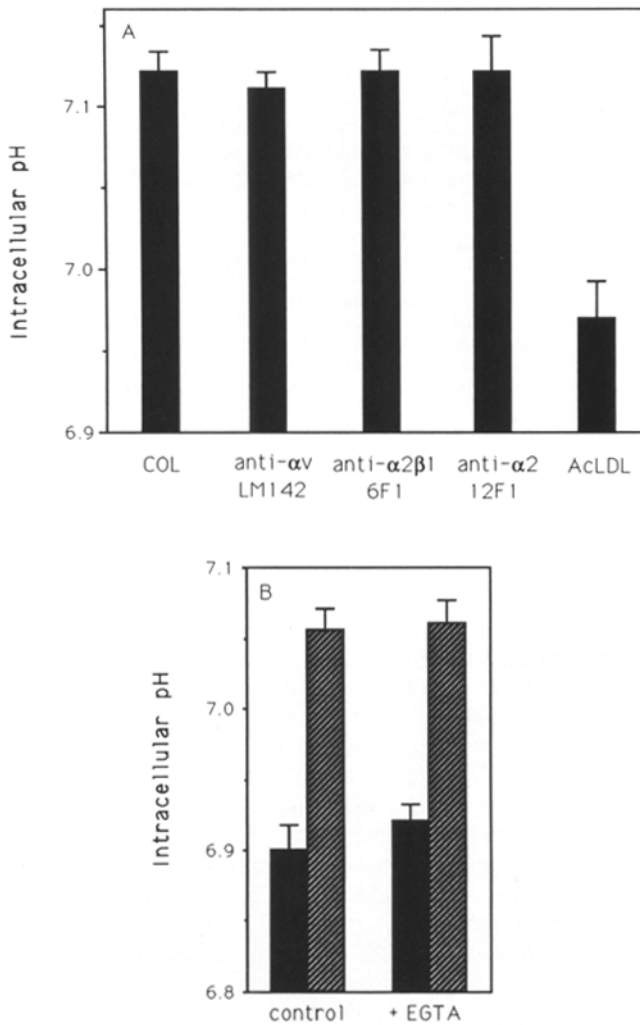
Figure 8. Effect of collagen, vitronectin, or anti-integrin antibodies on pH$_i$. (*A*) HUVECs loaded with BCECF were plated on 35-mm dishes coated in small areas with collagen or anti-integrin antibodies. After 90 min, pH$_i$ was determined, as described in Materials and Methods, in at least 15 cells within each area. Each bar represents the mean ± SD of at least 15 cells. (*B*) Cells were allowed to attach to vitronectin (▨) or AcLDL (■) and pHi was measured as above in the presence or absence of EGTA.

Figure 9. Effect of variable extracellular calcium on cell migration and intracellular calcium. HUVECs were suspended in adhesion buffer containing 1.8 mM calcium and magnesium and exogenously added EDTA. The exogenous level of calcium was calculated by subtracting the level of EGTA added from 1.8 mM calcium assuming a stoichiometry of one. In the upper panel cells were allowed to migrate toward vitronectin (□) or collagen (■) as described in Materials and Methods. Each data point represents the mean ± SD of four replicates. The lower panel represents the level of intracellular calcium measured on cells attached to vitronectin as described in Materials and Methods.

site for migration (Leavesley et al., 1992). Thus, it is likely that signaling events which regulate cell shape change or actin assembly may be different from those which initiate cell migration.

To address these issues, we examined the role of calcium in endothelial cell spreading and migration on a collagen or vitronectin matrix mediated by integrins $\alpha_2\beta_1$ and $\alpha_v\beta_3$, respectively. Cell migration was measured in the absence of growth factors, cytokines, or other soluble chemoattractants thereby permitting us to directly examine integrin-mediated cell motility. Cell attachment, spreading and elevation of pH$_i$ was found to be calcium independent on both matrix proteins. i.e., supported by magnesium alone. Surprisingly, cell migration to collagen was also independent of calcium. Migration on vitronectin, however, absolutely required extracellular calcium, being completely inhibited by treatment

with EGTA. We found that attachment to immobilized vitronectin or anti-$\alpha_v\beta_3$ IgG promoted a sustained rise in [Ca^{2+}]$_i$ that required a source of extracellular calcium, whereas collagen or $\alpha_2\beta_1$-specific mAbs failed to promote this response. The inhibitory effects of EGTA-treatment in these experiments were rapidly overcome when cells were returned to a buffer containing calcium demonstrating the effects of calcium chelation are reversible and nontoxic. When the cells are stimulated with growth factors or cytokines they typically show calcium levels several fold higher than observed due to vitronectin adhesion in our experiments (25–35 nm). This may be explained by the possibility that the local calcium concentration at the focal contact is actually considerably higher than that observed in the whole cell as measured in these experiments. While $\alpha_v\beta_3$

and $\alpha_2\beta_1$ apparently promote differential calcium signals, both integrins were capable of triggering an increase in pH_i upon ligand binding in the presence or absence of calcium. Collectively these data demonstrate that integrins $\alpha_v\beta_3$ and $\alpha_2\beta_1$ promote cellular migration through distinct signaling mechanisms. These data also provide the first evidence that cell spreading and migration do not necessarily share common signaling pathways.

Cell spreading depends upon cell attachment to an immobilized substrate supporting reorganization of the actin–cytoskeleton, whereas cell migration further requires detachment (de-adhesion) from the substrate. Thus, one possible explanation for our results may be that cell motility requires signaling events that promote detachment of integrin contacts. This may be regulated by calcium-dependent kinases or phosphatases. For example, integrin-dependent phosphorylation events, stimulated by receptor clustering and cell spreading (Kornberg et al., 1991; Guan et al., 1991), may stabilize focal adhesion structures. A dephosphorylation event might subsequently be required to destabilize this structure allowing cells to move (Tapley et al., 1989; Horvath et al., 1990). A recent study of neutrophil migration on vitronectin demonstrated the requirement for activation of a calcium-dependent phosphatase (calcineurin) to release cells from their adhesive contacts (Hendey and Maxfield, 1992). Thus, inhibitors of either calcineurin, or the calcium signal, prevented cell motility without affecting cell attachment or spreading (Hendey et al., 1992). To this end, the recent observation of localized increases in $[Ca^{2+}]_i$ in the trailing edge of migrating eosinophils (Brundage et al., 1991) suggests $[Ca^{2+}]_i$ may influence the stability of substrate adhesions. These data are consistent with our observations that vitronectin-mediated spreading is calcium independent whereas vitronectin-mediated migration is calcium dependent. Other mechanisms facilitating the release of cells into a migratory state could involve decreasing the highly stabilized ligand–integrin interaction (Orlando and Cheresh, 1991) via proteolysis of the matrix at sites of cell attachment (Werb et al., 1989; Seftor et al., 1992), or dissociation of integrin–cytoskeletal interactions, either of which could be triggered by $[Ca^{2+}]_i$. Alternatively, $[Ca^{2+}]_i$ may play a role in the cell's ability to sense the gradient of vitronectin or in establishing a polarized cytoskeleton. In any case, further research will be required to establish the mechanism by which calcium acts.

It is of interest that cell migration on collagen was independent of calcium implying that the mechanism of endothelial cell motility towards collagen is qualitatively different to that seen towards vitronectin. This may be accounted for by differences in the ligand binding properties of $\alpha_2\beta_1$ and $\alpha_v\beta_3$ or by different intracellular interactions of the cytoplasmic tails of these receptors with cytoskeletal proteins.

In summary, results from this study demonstrate that the integrins $\alpha_v\beta_3$ and $\alpha_2\beta_1$ expressed on endothelial cells promote distinct signaling events. Vitronectin recognition by $\alpha_v\beta_3$ promotes cellular migration in a calcium-dependent manner while collagen migration, mediated by $\alpha_2\beta_1$, occurs in the absence of calcium even though cellular spreading on both ligands is calcium independent. Thus, future studies will be designed to elucidate the integrin-stimulated signaling pathways and the underlying molecular events potentiating the migratory phenotype.

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