Spreading of Human Endothelial Cells on Fibronectin or Vitronectin Triggers Elevation of Intracellular Free Calcium

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Abstract. Intracellular calcium ($[Ca^{2+}]_i$) was measured in FURA 2-loaded endothelial cells plated on fibronectin or vitronectin. Average values for $[Ca^{2+}]_i$ increased to ~twofold above basal levels by ~1 h after plating, and then declined. The increase in $[Ca^{2+}]_i$ required extracellular calcium. Substituting potassium for sodium in the medium reduced the elevation of $[Ca^{2+}]_i$, a result that rules out the involvement of Na^+-Ca exchangers or voltage-dependent calcium channels, but that is consistent with the involvement of voltage-independent calcium channels. Plating cells on an anti-integrin β_1 subunit antibody gave a similar $[Ca^{2+}]_i$ response, but clustering β_1 integrins with the same antibody, or occupying integrins with RGD (arg-gly-asp) peptides had no effect. Time course measurements on single cells revealed that in each cell $[Ca^{2+}]_i$ rose abruptly at some point during spreading, from the basal level to a higher steady-state level that was maintained for some time. The elevated $[Ca^{2+}]_i$ was unrelated to previously observed changes in intracellular pH, because chelating the Ca^{2+} in the medium failed to inhibit the elevation of pHi that occurred during cell spreading. In conclusion, these results show that integrin-mediated cell spreading can regulate $[Ca^{2+}]_i$, and the pathways involved are distinct from those that regulate intracellular pH.

FIBRONECTIN (FN)[1] and other extracellular matrix (ECM) proteins not only mediate cell adhesion and spreading, but can regulate a wide range of cell functions, such as growth of anchorage-dependent cells, activation of leukocytes and platelets, gene expression and differentiation in many cell types (reviewed in Donjacour and Cunha, 1991; Shimizu and Shaw, 1991; Ingber and Folkman, 1989). These regulatory effects appear to be exerted, at least in part, through effects of integrin receptors on signaling pathways similar to those used by hormone and growth factor receptors (reviewed in Hynes, 1992). Such pathways include tyrosine phosphorylation, intracellular pH, and inositol lipid turnover.

My laboratory has shown that spreading of cells on FN, or clustering of integrins by antibodies (Schwartz et al., 1991*a*, *b*; Ingber et al., 1990), induces an increase in intracellular pH by activating the Na-H antiporter. In C3H 10T1/2 cells, a mouse fibroblast line, some of the effect on pHi has been shown to be due to a protein kinase C (PKC)-dependent pathway involving synergy between FN and serum or PDGF (Schwartz and Lechene, 1992). In endothelial cells, however, activation of the Na-H antiporter is independent of both soluble growth factors and of PKC, indicating that another pathway(s) must mediate the effect of integrins. In several other systems, intracellular calcium has been shown to activate the antiporter and elevate pHi via Ca^{2+}/calmodulin-dependent protein kinase (Manganel and Turner, 1990; Kimura et al., 1990; Ober and Pardee, 1987). These results prompted me to investigate whether intracellular calcium is controlled by adhesion in endothelial cells, and whether it mediates effects on intracellular pH.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; FN, fibronectin; VN, vitronectin.

Materials and Methods

Chemicals and Biochemicals

BCECF-AM, FURA2, FURA2-AM and the bis oxonol dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol were obtained from Molecular Probes Inc. (Eugene, OR). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Proteins

Fibronectin was prepared from human plasma by affinity chromatography on gelatin-Sepharose (Miekka et al., 1982). Antibody TS2/16 (IgG1) to the integrin β_1 chain was a gift from Dr. Martin Hemler (Harvard Medical School, Boston, MA). Affinity purified goat anti-mouse IgG was purchased from Sigma Chemical Co. FITC-conjugated affinity purified goat anti-mouse IgG was from Cappel Laboratories (Malvern, PA). Antibody W6/32 (IgG2a) to HLA and vitronectin (VN) were gifts from Dr. David Cheresh (The Scripps Research Clinic, La Jolla, CA). Antibody R6.5 (IgG 2a) to ICAM-1 was a gift from Dr. Michael Lawrence (University of Washington, Seattle, WA), and antibody 489 to VCAM-1 (IgG1) was from Dr. John Harlan. Acetylated low density lipoprotein was purchased from Biomedical Technologies Inc. (Stoughton, MA).

Coverslips

Wells in 35-mm dishes were manufactured by cutting a hole in the bottom of the dish and attaching a coverslip to the outside of the dish with silicone

grease. The dishes were coated with FN (40 μg/ml), VN (40 μg/ml), or acetylated LDL (100 μg/ml) for 1 h, then rinsed and blocked with 1% heat denatured BSA in PBS. For antibodies, the coverslips were coated first with 50 μg/ml anti-mouse IgG for 1 h, blocked with 1% heat-denatured BSA, then incubated with 10–20 μg/ml of specific antibody in 1% heat-denatured BSA for 1 h.

Cells

Human umbilical vein endothelial cells were generous gifts of Drs. David Loskutoff or Eugene Levin (The Scripps Research Institute, La Jolla, CA). Cells were subcultured by trypsinization, and were plated on tissue culture plastic in either EGM-UV medium (Clonetics Corp., San Diego, CA) or medium 199 (Gibco Laboratories, Grand Island, NY) supplemented with 20% FBS (GIBCO Laboratories), 50 μg/ml ECGS (Upstate Biotechnology, Saranac Lake, NY) and 5 μg/ml heparin (Sigma Chemical Co.). Cells were used between passages 2 and 10.

For calcium and pH experiments, cells were detached by either of two methods. First, cells were incubated at 37°C in DME with 10 mM EDTA and 0.20 M urea (Levin and Santell, 1987). After 2-3 min, cells were completely rounded, and were detached from the dish by gentle pipetting. Alternatively, cells were incubated in PBS containing 2 mM EDTA for 5 min, then detached by pipetting. We found that cells released by either method spread faster, had lower baseline calcium levels, and improved calcium responses to thrombin and histamine compared to cells released with trypsin. Cells detached by either method gave similar results.

The cells were collected by low speed centrifugation and resuspended in basal medium consisting of DME with 0.1% protease- and endotoxin-free BSA (CalBiochem Corp., San Diego, CA), and 1% growth medium supplement G, containing insulin, selinium, and transferrin (GIBCO BRL, Gaithersburg, MD). These additions improve cell viability over the 4-8 h involved in these experiments, but do not appear to directly affect intracellular calcium or pH. After detachment, cells were held in suspension by plating them in bacterial plastic dishes coated with 1% BSA that had been denatured by heating to 90°C for 10 min. Cells were incubated in suspension for 2-6 h to induce quiescence before carrying out calcium or pHi measurements.

Intracellular Calcium and pHi Measurements

For measurements of intracellular calcium, cells were incubated with 2-4 μM FURA2-AM for 20-40 min, rinsed and plated in the dishes on the stage of a Nikon diaphot microscope, equipped with the Photoscan 2 system for dual excitation microscopy. This system employs a xenon arc light source, the light from which passes through a variable diaphragm, then is directed by a chopper alternating at 100 Hz through either of two band pass filters. The band pass filters (Omega Optics, Brattleboro, VT) have 20-nm bandwidths, and are centered on 340 and 380 nm. The light is directed onto the sample through fiber optic cables, then through a 485-nm dichroic mirror and a 20× quartz 0.75 N.A. Nikon Fluor objective onto the cells. Emitted light passes through a second diaphragm that selects the light from a small portion of the field. 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 emitted light then passes through a 510-nm band pass filter (bandwidth 20 nm) and into the photon counter.

The system is operated by a NEC 286 IBM clone computer, using Photoscan 2 software. Background measurements were made from nearby cell-free areas, and fluorescence intensities corrected for background. The 380/340 ratio was then calculated, and intracellular calcium was estimated using the equation:

$$[Ca^{2+}]_i = Kd \times \frac{R - R_{min}}{R_{max} - R} \times \frac{Sf2}{Sb2}.$$

Values for R_{min} and Sf2 were determined by measuring the fluorescence from 1 μM FURA2 in 100 mM KCl, Hepes pH 7.2, with 10 mM EGTA for <10 nM Ca^{2+}. Sb2 was determined in this solution plus 10 mM $CaCl_2$. R_{max} was determined by adding 10 μM ionomycin to cells in DME, and was close to the value obtained from cell-free FURA2 with 10 mM calcium, indicating that hydrolysis of the AM ester was complete. Kd was taken to be 224 nM, for cells at 37°C. $[Ca^{2+}]_i$ calculated in this manner agreed well with free Ca^{2+} determined experimentally using buffered solutions of $CaCl_2$ and EGTA in 100 mM KCL, 20 mM Hepes pH 7.2 (not shown).

For continuous time course measurements on single cells, the first diaphragm was used to reduce the excitation light to the minimal intensity that gave satisfactory signal-to-noise ratios. For population measurements, each cell was measured for ~10 s, with ~15 cells measured in quick succession. Data is shown as the mean ± standard error.

For pHi measurements, the same dual excitation protocol was employed but with different filters. The two excitation filters were centered at 440 and 490 nm, the emission filter was center at 530 nm (all 20 nm bandwidth, obtained from Chroma Technologies, Brattleboro, VT), with a 515 nm dichroic mirror (Chroma Technologies). The measurements were calibrated using high potassium buffer and nigericin as we have described previously (Ingber et al., 1990). Each cell was measured for 10 s, and values are means ± standard errors for ~15 cells per data point.

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 that was heated by water from a recirculating waterbath. Air from an aquarium air pump was mixed with CO_2 using a mixing chamber, humidified by bubbling through warm deionized water, passed through a stainless steel tube surrounded by a heating element, and directed into the chamber where it blew across the surface of the medium. The heating element was controlled by a YSI model 72 temperature controller, and monitored by a YSI 511 temperature probe that was placed directly in the medium. This system gave constant temperature (±<0.5°C), without overshoots at any time. Temperature was set at 37°C and CO_2 was set at 7.5%.

Results

Intracellular Calcium During Cell Spreading

The first question to be addressed was whether plating endothelial cells on FN had any effect on intracellular calcium $[Ca^{2+}]_i$. Cells in suspension were loaded with the calcium indicator FURA2, then plated on FN-coated glass coverslips on the microscope stage. Cells attached in <5 min under these conditions, spread in a symmetrical manner over 30–60 min, and polarized over several hours. When $[Ca^{2+}]_i$ was measured in populations of cells at several time points after plating, a slow rise in intracellular calcium was observed (Fig. 1 *A*). Average values for $[Ca^{2+}]_i$ were elevated ~twofold over basal levels at 1 h, and then slowly declined. Cells plated on BSA failed to spread and showed no change in $[Ca^{2+}]_i$ over this time period (not shown). The rise in $[Ca^{2+}]_i$ appeared to coincide with symmetrical spreading, and the decline with polarization of the cells. Similar results were obtained on VN, though the rate of spreading and polarization were somewhat higher, and the rise and fall in $[Ca^{2+}]_i$ were correspondingly faster (Fig. 1 *B*).

Involvement of Calcium Channels

To determine whether the elevation of calcium was due to release from internal stores or to influx through the plasma membrane, 10 mM EGTA was added to the medium of cells plated on FN. As a control, cells were also plated on acetylated LDL (AcLDL), to which they adhered via the scavenger receptor, but did not spread. Chelation of extracellular calcium led to a decline in $[Ca^{2+}]_i$ in cells spread on FN that was complete in 20 s (not shown). Measurements of average $[Ca^{2+}]_i$ revealed that EGTA completely abolished the adhesion-dependent rise in $[Ca^{2+}]_i$, but on this time scale had no effect on $[Ca^{2+}]_i$ in cells on AcLDL (Fig. 2 *A*). This result suggested that calcium channels in the plasma membrane might be responsible for the elevation in $[Ca^{2+}]_i$, though it is also possible that other mechanisms such as Na-Ca exchange are involved.

To test both the participation of Na-Ca exchange and the voltage dependence of any calcium channels, cells that had spread in buffer containing normal sodium were transferred to buffer with up to 122 mM potassium (keeping osmolarity constant). This concentration was chosen because it should

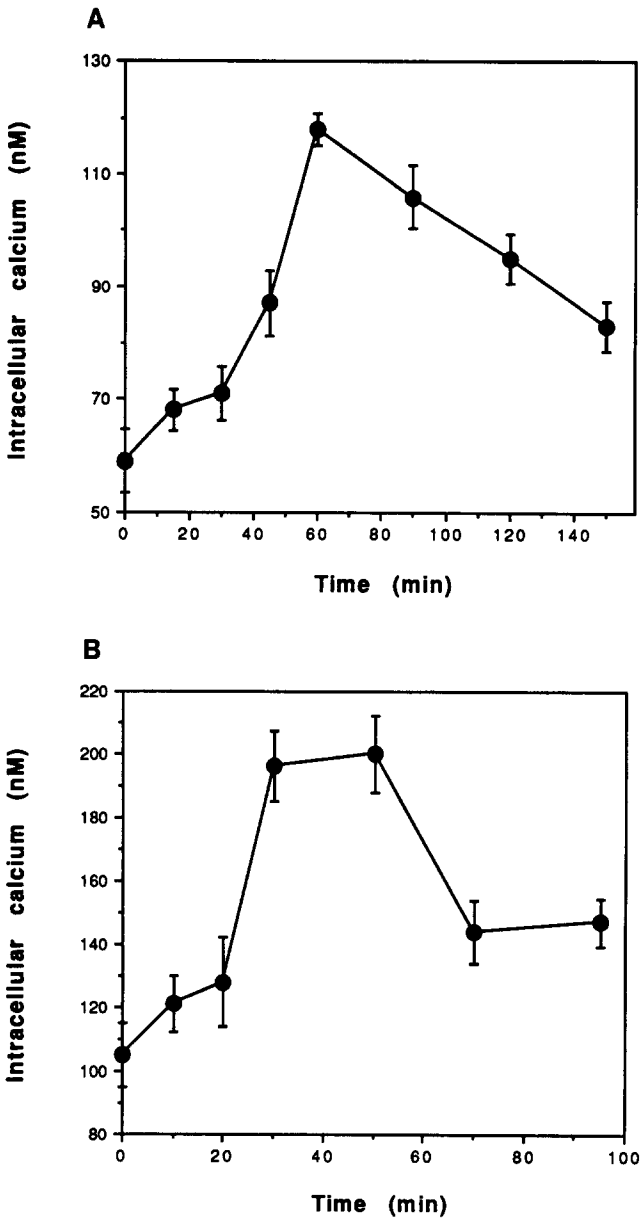

Figure 1. $[Ca^{2+}]_i$ in populations of spreading cells. $[Ca^{2+}]_i$ was measured in ~15 cells at the indicated times, and the means ± standard error calculated. (*A*) Cells plated on FN; (*B*) cells plated on VN. Similar results were obtained in at least three experiments for each protein.

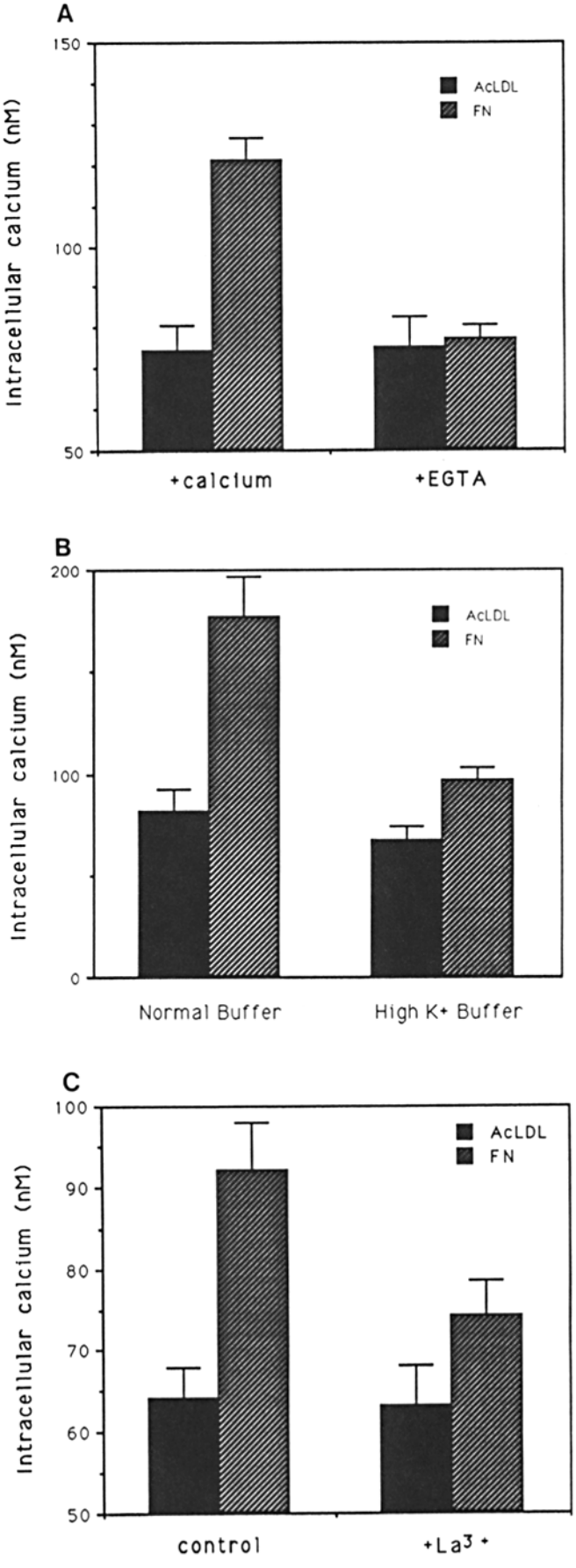

Figure 2. (*A*) Effect of EGTA on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in cells plated for 60 min on FN or acetylated LDL (AcLDL) in standard medium, then 10 mM EGTA and 4 mM $MgCl_2$ were added, and $[Ca^{2+}]_i$ measured. Similar results were obtained in six experiments. (*B*) Effect of high K^+ on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in cells plated for 60 min on FN or AcLDL, in buffer containing 10 mM Hepes pH 7.35, 135 mM NaCl, 5 mM KCL, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM glucose. The buffer was then removed, new buffer added which had the same composition except that 120 mM KCl was isotonically substituted for NaCl, and $[Ca^{2+}]_i$ measured again. Similar results were obtained in three experiments. (*C*) Effect of La^{3+} on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in cells plated 60 min on FN or AcLDL in Hepes-buffered saline, then 0.25 mM $LaCl_3$ added and $[Ca^{2+}]_i$ measured again. Similar results were obtained in four experiments.

induce nearly complete depolarization, but sufficient sodium was still available to maintain the activity of the Na-H antiporter, so that intracellular pH would not be altered (Ingber et al., 1990; Schwartz et al., 1989). If voltage-dependent calcium channels were present, this procedure should trigger depolarization and an influx of calcium. If Na-Ca exchangers were present, the reduction in extracellular Na^+ should also trigger an influx of Ca^{2+} (Goodinich et al., 1991). Upon transfer to high potassium buffer, no transient changes in $[Ca^{2+}]_i$ were detected (not shown). In parallel experiments, membrane depolarization could be detected by a large increase in the fluorescence of a membrane potential-sensitive bisoxonol dye (Lakos, 1990) (data not shown). Measurements of average $[Ca^{2+}]_i$ revealed that the adhesion-dependent elevation of $[Ca^{2+}]_i$ was diminished in high potassium (Fig. 2 *B*). The calcium elevation in 122 mM K^+ was found to be 36 ± 12% of control values (n=3). These results rule out the involvement of both Na-Ca exchange and voltage-dependent calcium channels in the adhesion-dependent increase in $[Ca^{2+}]_i$. Moreover, the data is consistent with work showing that calcium entry through voltage-independent calcium channels is decreased by depolarization, due to the fact that membrane potential provides a significant portion of the driving force for entry of calcium ions. Theoretical calculations and measurements of calcium entry both indicate that depolarization diminishes calcium fluxes by a factor of ~3 (Curry, 1992), in agreement with the data obtained here. Results with high K^+ therefore support the notion that calcium enters from the extracellular medium.

Voltage-independent, receptor-activated calcium channels are generally unaffected by the dihydropyridine antagonists. Consistent with this prediction, neither verapamil nor diltiazem (both at 100 μM) inhibited the rise in $[Ca^{2+}]_i$ on FN (not shown). Voltage-independent calcium channels can usually be inhibited by heavy metal ions, however. For example, histamine-responsive calcium channels in endothelial cells are inhibitable by Ni^{2+} and La^{3+} (Curry, 1992). I confirmed that adding 0.2 mM $NiCl_2$ or 0.25 mM $LaCl_3$ to cells 5 min after histamine stimulation caused a rapid decline of $[Ca^{2+}]_i$ to baseline levels (not shown). When these metal ions were added to cells spreading on FN, however, 0.2 mM Ni^{2+} had no effect on $[Ca^{2+}]_i$ levels (not shown). La^{3+} (0.25 mM) gave partial inhibition (Fig. 2 *C*). Higher concentrations had no further effect. These results indicate that the adhesion-activated calcium channels are distinct from those regulated by other agonists.

$[Ca^{2+}]_i$ in Single Cells

The data shown in Figs. 1 and 2 represent averages from multiple cells, which may not accurately reflect the behavior of individual cells within the population. Therefore, continuous measurements were made of $[Ca^{2+}]_i$ in single cells after plating. Fig. 3 shows typical time courses for cells plated on FN, and a control time course for a cell on BSA. Instead of a slow increase in $[Ca^{2+}]_i$ that parallels the increase in cell area, most cells had a steady basal level of $[Ca^{2+}]_i$ during the initial phase of cell spreading, with a relatively rapid shift to a new, higher level that remained steady for the duration of the experiment. Cells on VN showed a similar pattern (Fig. 4). Both the timing and the magnitude of the change in $[Ca^{2+}]_i$ varied substantially between individual cells. The shift in $[Ca^{2+}]_i$ occurred after cells had attached and were at least partially spread, but could occur at any point during cell spreading. Though the timing varied, the overall pattern was reproducible, being observed in 17 out of 21 cells. No obvious change in morphology was observed to correlate with the shift in $[Ca^{2+}]_i$. Because illumination of FURA-loaded cells is eventually toxic, it was not feasible to observe single cells long enough to observe the decline in $[Ca^{2+}]_i$ that occurred at later times.

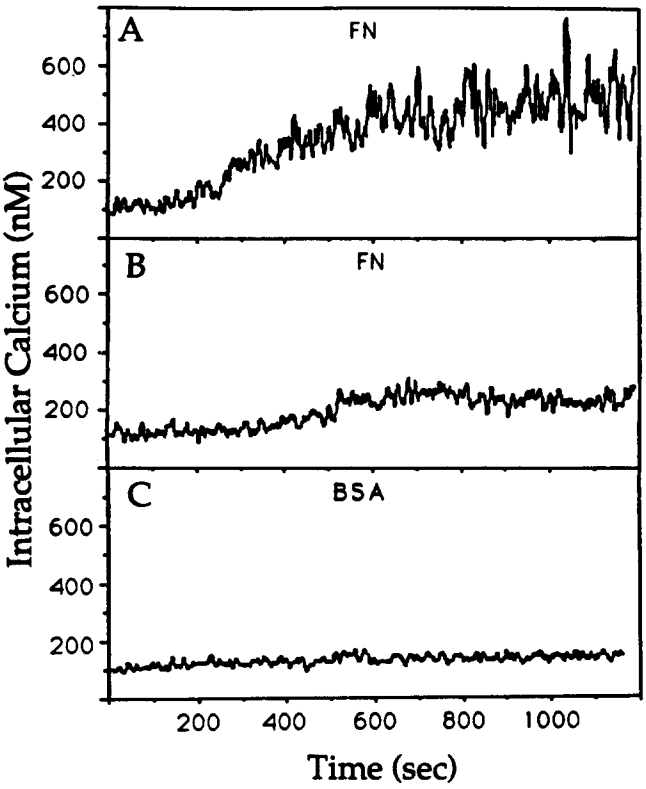

Figure 3. Intracellular calcium in single cells on FN. Cells were plated on FN (*A* and *B*) or BSA (*C*), and $[Ca^{2+}]_i$ followed in single cells during cell spreading.

Role of Integrins

To determine whether integrins mediated the observed effects, cells were plated on coverslips coated with a monoclonal antibody to the integrin β_1 subunit. Cells adhered and spread under these conditions much as they do on FN (Fig. 5). When FURA2-loaded cells were plated on anti-β_1 IgG, $[Ca^{2+}]_i$ was observed to show a transient increase similar to cells on FN (Fig. 6 *A*). This elevation of $[Ca^{2+}]_i$ was eliminated or greatly reduced by adding EGTA to the medium (Fig. 6 *B*). Cells plated on anti-HLA IgG adhered, but did not spread, and showed no change in $[Ca^{2+}]_i$ (not shown), indicating that the effect on $[Ca^{2+}]_i$ is specific to anti-integrin IgG. Cells on antibodies to the VN receptor, integrin $\alpha_v\beta_3$, also showed a transient rise in $[Ca^{2+}]_i$ (unpublished data). These results suggest that integrins are able to transduce the observed effects on $[Ca^{2+}]_i$.

As additional controls, the effect of adhesion to anti-ICAM-1 and anti-VCAM-1 on $[Ca^{2+}]_i$ was examined. Resting human endothelial cells have modest levels of ICAM-1 (~10^5 copies/cell) which are increased 50-fold by treatment with interleukin 1α (IL-1α) (Dustin and Springer, 1988), so that ICAM-1 levels are similar to the β_1 integrin subunit.

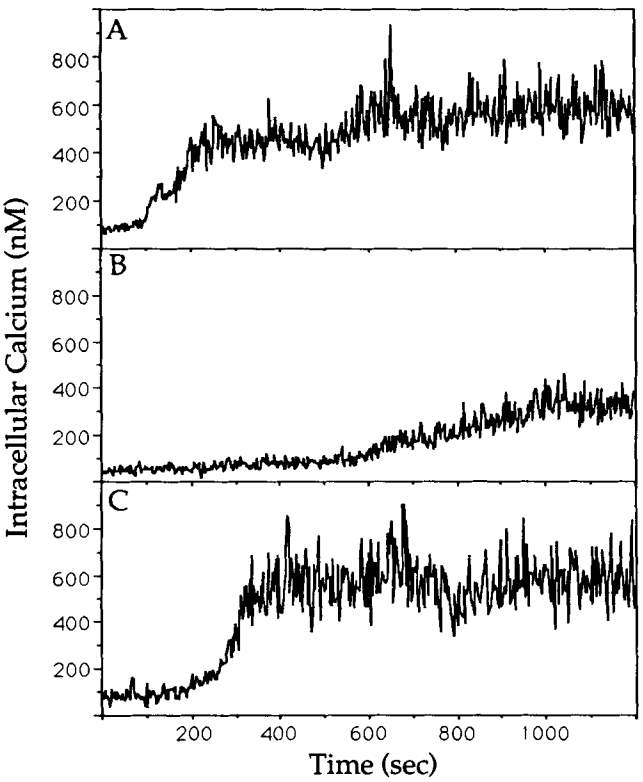

Figure 4. Intracellular calcium on VN. Cells were plated on VN and $[Ca^{2+}]_i$ followed as a function of time, similar to Fig. 3. (*A*) Two cells in a cluster undergo abrupt increases at slightly different times; (*B*) one of the minor fraction of cells where the increase in $[Ca^{2+}]_i$ does not occur rapidly. These represent <20% of the population; (*C*) a cell undergoes a rapid increase in $[Ca^{2+}]_i$.

VCAM-1 is also induced by IL-1α. I found that uninduced cells adhered to some extent but did not spread on coverslips coated with the anti-ICAM-1 mab R6.5, or with the anti-VCAM-1 mab 489 (which has the same isotype as the anti-β1 mab). Treatment with 50 ng/ml IL-1α for 24 h caused the expected dramatic increase in ICAM-1 and VCAM-1 expression, as detected by immunofluorescence (not shown). Induced cells showed greatly enhanced adhesion to anti-VCAM-1 though no spreading occurred. On anti-ICAM-1, both enhanced adhesion and spreading were observed. When $[Ca^{2+}]_i$ was monitored during adhesion of IL-1α treated cells to these antibodies, no changes could be observed during 1 h of adhesion or spreading (not shown). These results demonstrate that the effect of the anti-β_1 integrin mab is specific.

Previous work has shown that clustering β_1 integrins with soluble antibodies is sufficient to induce activation of the Na-H antiporter (Schwartz et al., 1991*a*) and phosphorylation of proteins on tyrosine (Kornberg et al., 1991). In addition, clustering of β_2 integrins on leukocytes with soluble antibodies has been shown to induce elevations in $[Ca^{2+}]_i$ (Ng-Sikorski et al., 1991; Pardi et al., 1989). The effect on $[Ca^{2+}]_i$ of clustering the β_1 integrin was therefore examined.

Cells were incubated in the cold with anti-β_1 IgG, then rinsed and incubated with second antibody. Fig. 7 shows that when FITC-conjugated second antibody was used, rapid patching and capping could be observed upon warming to 37°C. Parallel preparations, however, showed no change in $[Ca^{2+}]_i$ on this time scale (Fig. 8). This procedure was previously found to trigger an increase in pHi using the same antibody (Schwartz et al., 1991*a*). Experiments in which cells were maintained at 37°C and clustering induced by addition of anti-integrin Fab fragments followed by second antibody also failed to induce a detectable change in $[Ca^{2+}]_i$ (not shown).

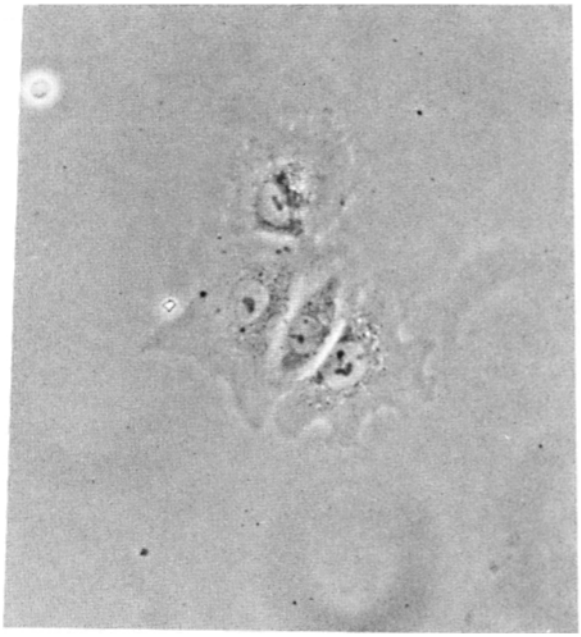

Figure 5. Cells spreading on anti-integrin IgG. Cells were plated on a coverslip coated with monoclonal antibody TS2/16 to the integrin β_1 subunit, and photographed under phase contrast optics at 1 h.

We have also attempted to trigger calcium increases by occupying integrins with RGD peptides. Addition of 0.5 mM of the peptide GRGDSP, which completely blocks spreading of cells on FN or VN, failed to induce any change in $[Ca^{2+}]_i$ when added to suspended cells (data not shown). Thus, neither receptor clustering nor occupancy is sufficient for calcium entry in the absence of cell spreading.

Effect of $[Ca^{2+}]_i$ on pHi

To determine whether the elevation of $[Ca^{2+}]_i$ during spreading was responsible for the change in pHi that occurs when cells spread on FN, EGTA was added to the medium to block the rise in $[Ca^{2+}]_i$. To prevent inhibition of spreading itself, an additional 4 mM Mg^{2+} was added to the medium. Under these conditions, cells spread normally. Fig. 9 shows that pHi was elevated in cells on FN equally well with or without Ca^{2+} in the medium. Similar results were obtained with vitronectin (not shown). $[Ca^{2+}]_i$ measurements confirmed that $[Ca^{2+}]_i$ declined slowly during the time course of spreading (not shown). These results demonstrate that the elevation of pHi is not mediated by changes in $[Ca^{2+}]_i$ in these cells.

Discussion

Our results show that cell spreading on FN or VN is accompanied by an elevation of $[Ca^{2+}]_i$, which requires the pres-

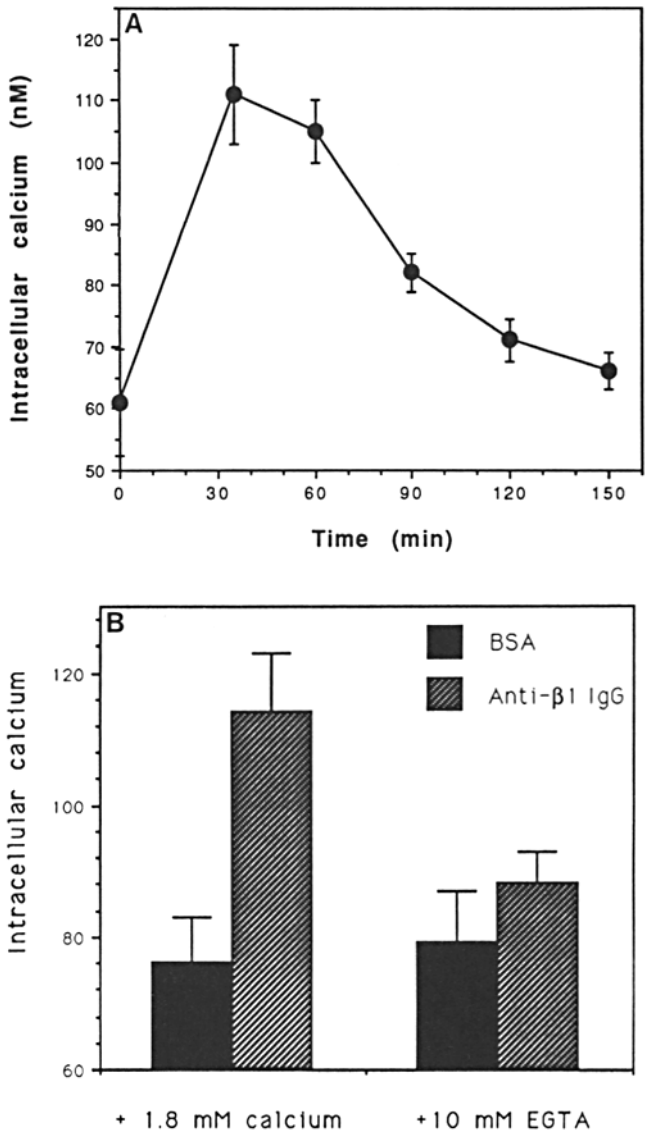

Figure 6. $[Ca^{2+}]_i$ in cells plated on anti-integrin IgG. (*A*) Cells were plated on TS2/16 anti-β_1 integrin IgG, and $[Ca^{2+}]_i$ measured in ~15 cells at each time point. Similar results were obtained in four experiments. (*B*) $[Ca^{2+}]_i$ was measured in cells plated for 60 min on anti-β_1 IgG or on BSA, then EGTA was added and $[Ca^{2+}]_i$ measured again. Similar results were obtained in three experiments.

ence of Ca^{2+} in the medium. $[Ca^{2+}]_i$ levels peaked around 1 h after plating, and then declined slowly. Substitution of potassium for sodium in the medium failed to induce an influx of calcium into the cells, which rules out the involvement of both voltage dependent calcium channels and Na-Ca exchange. Blockade by La^{3+} supports the notion that calcium channels in the plasma membrane are probably involved.

Integrins appear to be involved in triggering the elevation of $[Ca^{2+}]_i$, because plating cells on immobilized anti-integrin IgG also induced transient increases in $[Ca^{2+}]_i$ that required extracellular Ca^{2+}. Cross-linking of integrins with soluble antibodies did not, however, induce a detectable rise in $[Ca^{2+}]_i$. This point may be of some interest, because the leukocyte integrins LFA-1 and Mac-1 have both been shown

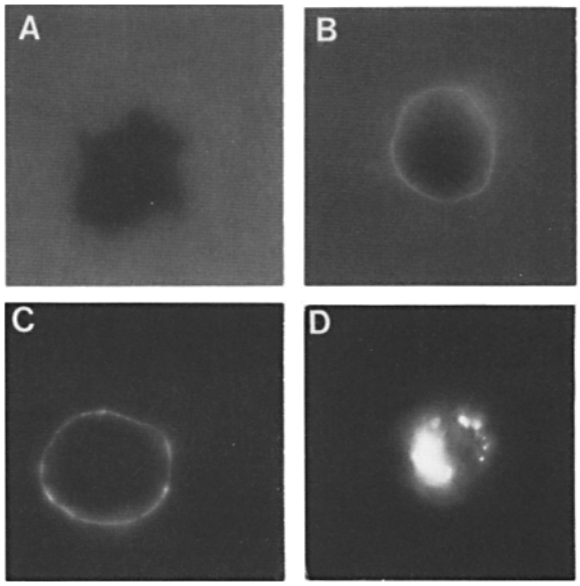

Figure 7. Integrin clustering and capping. Cells were incubated with 10 μg/ml TS2/16 anti-β_1 IgG on ice, followed by 10 μg/ml second antibody, then warmed to 37°C. (*A*) no first antibody; (*B*) cells fixed before antibody incubations; (*C*) cells fixed after antibody incubations; (*D*) cells warmed to 37°C for 10 min after antibody incubations, then fixed.

to induce large calcium transients when cross-linked with soluble antibodies (Ng-Sikorski et al., 1991; Pardi et al., 1989). Mac-1-mediated calcium transients have been linked to migration of neutrophils (Ng-Sikorski et al., 1991). The fact that cross-linking of β_1 integrins failed to trigger $[Ca^{2+}]_i$ transients, together with the distinct characteristics of the changes in $[Ca^{2+}]_i$ in the two systems, argue that the effects in leukocytes and endothelial cells may represent distinct phenomena. The results may also indicate that the cytoskeleton plays a role in the opening of calcium channels in the

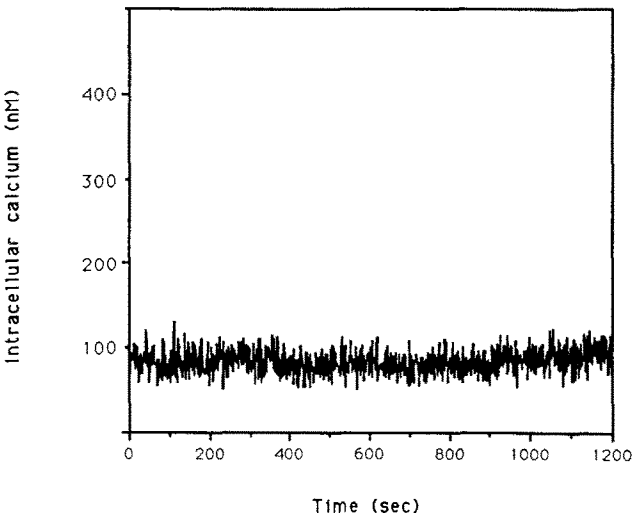

Figure 8. Effect of integrin clustering on intracellular calcium. Cells were incubated at 4°C with TS2/16 followed by second antibody as in Fig. 7. Cells were than transferred to the microscope stage at 37°C and $[Ca^{2+}]_i$ followed in a cluster of eight cells. Similar results were obtained in eight experiments.

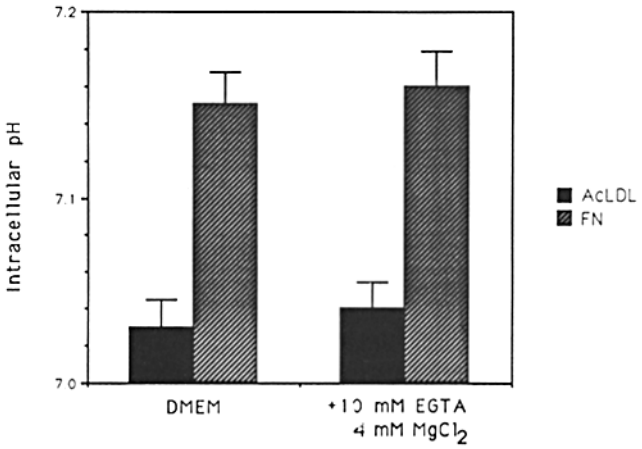

Figure 9. Effect of medium calcium on pHi. Cells were plated on AcLDL or FN for 2 h in normal medium or medium to which 10 mM EDTA and 4 mM $MgCl_2$ had been added, and intracellular pH measured. Similar results were obtained in three experiments.

plasma membrane. It is also possible, however, that clustering is sufficient, but that internalization of the cross-linked complexes blocks the rise in calcium in this system. Cross-linked integrins have been shown to be rapidly internalized, leading to termination of the pHi and phosphorylation signals (Schwartz et al., 1991*a*; Kornberg et al., 1991). If calcium channels are activated locally, only in proximity to the integrins, internalization could explain the failure to observe a detectable rise in $[Ca^{2+}]_i$. Internalization could stop calcium entry by removing the calcium channels from either the source of calcium (the extracellular milieu) or the source of activation (the integrins), depending upon whether the channels were cointernalized. Calcium might therefore be more sensitive to internalization than other integrin-mediated signaling events.

Measurements of $[Ca^{2+}]_i$ in single cells during spreading revealed that individual cells underwent relatively abrupt changes in $[Ca^{2+}]_i$. Whereas cell spreading occurs over 30–60 min, the majority of cells showed $[Ca^{2+}]_i$ increase that were complete in seconds to a few minutes. Cells then maintained high $[Ca^{2+}]_i$ levels for at least 10–20 min. The observation that a slow change in spreading can be converted to a rapid change in $[Ca^{2+}]_i$ deserves some comment. Cells must integrate the input in a way that produces a temporal "sharpening" of the signal. The mechanism by which this occurs remains to be determined, but autocatalysis, in which elevated calcium contributes to further entry of calcium seems likely.

By several criteria, the elevation of $[Ca^{2+}]_i$ does not appear to be related to the elevation of pHi that occurs when endothelial cells spread on ECM proteins. First, removal of extracellular calcium blocks the rise in $[Ca^{2+}]_i$, but does not block the elevation of pHi. Second, $[Ca^{2+}]_i$ and pHi have rather distinct time courses. Though they both increase as cells spread, pHi does so fairly smoothly (Ingber et al., 1990), whereas $[Ca^{2+}]_i$ does so abruptly. Furthermore, the elevation of pHi persists at later times, when $[Ca^{2+}]_i$ levels have decreased. Third, cross-linking of integrins, which we have observed induces a rise in pHi, failed to trigger a change in $[Ca^{2+}]_i$. It appears, therefore, that regulation of pHi and $[Ca^{2+}]_i$ by integrins are separable, and are likely to occur via distinct pathways.

The function of integrin-mediated changes in $[Ca^{2+}]_i$ remains unknown, but a role in cell migration is an attractive speculation. $[Ca^{2+}]_i$ is well-characterized as a modulator of the cytoskeleton, and appears to play a role in migration in other systems. Recent work (Grzesiak et al., 1992) has shown that migration of cells on VN is stimulated by calcium in the medium, under conditions where cell adhesion and spreading are independent of medium Ca^{2+}. Furthermore, it is tempting to speculate that the "sharpening" of the signal could be important for migration in response to gradients of ECM proteins (haptotaxis). Previous work has shown that cells exhibit directed migration on very shallow gradients of adhesive ligands. In one case, cells showed directional migration under conditions where the change in ligand concentration over a cell length was only 0.1%. Some means must exist for enhancing the cells' perception of the ECM protein gradients in these situations. Autocatalytic entry of Ca^{2+} is one such mechanism, but however it occurs, it seems likely that the temporal sharpening could be related to the spatial enhancement that must occur in haptotaxis. These possibilities are currently being investigated.

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