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

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Abstract. Intracellular calcium ([Ca\textsuperscript{2+}]i) was measured in FURA 2-loaded endothelial cells plated on fibronectin or vitronectin. Average values for [Ca\textsuperscript{2+}]i increased to twofold above basal levels by \(\sim 1\) h after plating, and then declined. The increase in [Ca\textsuperscript{2+}]i required extracellular calcium. Substituting potassium for sodium in the medium reduced the elevation of [Ca\textsuperscript{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 \(\beta_1\) subunit antibody gave a similar [Ca\textsuperscript{2+}]i response, but clustering \(\beta_1\) 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\textsuperscript{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\textsuperscript{2+}]i was unrelated to previously observed changes in intracellular pH, because chelating the Ca\textsuperscript{2+} in the medium failed to inhibit the elevation of pH that occurred during cell spreading. In conclusion, these results show that integrin-mediated cell spreading can regulate [Ca\textsuperscript{2+}]i, and the pathways involved are distinct from those that regulate intracellular pH.

Fibronectin (FN) 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., 1991a, 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 pH 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 pH via Ca\textsuperscript{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.

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 (Mieska et al., 1982). Antibody TS2/16 (IgG1) to the integrin \(\beta_1\) 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...
Intracellular Calcium and pH 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, light passes through a second diaphragm that selects the light from a small portion of the field. The emitted light then passes through a 510-nm band pass filter (bandwidth 20 nm) and into the photon counter.

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 0.2 M urea (Levin and Santall, 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, selenium, 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 placing 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 pH measurements.

Intracellular Calcium and pH 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-μm dichroic mirror and 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-μm dichroic mirror and a 20× quartz 0.75 NA. 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-μm band pass filter (bandwidth 20 nm) and into the photon counter.

The system is operated by a NBC 286 IBM clone computer, using the 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:

\[ [Ca^{2+}] = \frac{K_d \times R - R_{\text{min}} \times S_{2}}{R_{\text{max}} - R} \times S_{2}. \]

Values for \( R_{\text{max}} \) and \( S_{2} \) were determined by measuring the fluorescence from 1 μM FURA2 in 100 mM KCl, Hepes pH 7.2, with 10 mM EGTA for <10 mM Ca²⁺. \( S_{2} \) was determined in this solution plus 10 mM CaCl₂. \( R_{\text{max}} \) was determined by adding 10 μM ionomycin to cells in DME, and was close to the value obtained from cell-free FURA2 with 10 μM calcium, indicating that hydrolysis of the AM ester was complete. Kd was taken to be 224 nM, for cells at 37°C. [Ca²⁺] calculated in this manner agreed well with free Ca²⁺ determined experimentally using buffered solutions of CaCl₂ and EGTA in 100 mM KCl, 20 mM Hepes pH 7.2 (not shown).

For continuous time course measurements on single cells, the first diaphram 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 pH 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 centered 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 (Ingeber 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 water-bath. Air from an aquarium air pump was mixed with CO₂ 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 CO₂ 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 [Ca²⁺]. 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 [Ca²⁺], 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 [Ca²⁺], 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 [Ca²⁺], over this time period (not shown). The rise in [Ca²⁺], 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 [Ca²⁺], 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 [Ca²⁺], in cells spread on FN that was complete in 20 s (not shown). Measurements of average [Ca²⁺], revealed that EGTA completely abolished the adhesion-dependent rise in [Ca²⁺], but on this time scale had no effect on [Ca²⁺], in cells on AcLDL (Fig. 2 A). This result suggested that calcium channels in the plasma membrane might be responsible for the elevation in [Ca²⁺], 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. $[\text{Ca}^{2+}]_i$ in populations of spreading cells. $[\text{Ca}^{2+}]_i$ was measured in $\sim 15$ 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 $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ measured. Similar results were obtained in six experiments. (B) Effect of high K$^+$ on $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ measured again. Similar results were obtained in three experiments. (C) Effect of La$^{3+}$ on $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ was measured in cells plated 60 min on FN or AcLDL in Hepes-buffered saline, then 0.25 mM LaCl$_3$ added and $[\text{Ca}^{2+}]_i$ 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 (Inghber 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 Ca2+ (Goodinich et al., 1991). Upon transfer to high potassium buffer, no transient changes in [Ca2+] 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 [Ca2+] revealed that the adhesion-dependent elevation of [Ca2+] 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 [Ca2+]. 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 [Ca2+] 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 Ni2+ and La3+ (Curry, 1992). I confirmed that adding 0.2 mM NiCl2 or 0.25 mM LaCl3 to cells 5 min after histamine stimulation caused a rapid decline of [Ca2+], to baseline levels (not shown). When these metal ions were added to cells spreading on FN, however, 0.2 mM Ni2+ had no effect on [Ca2+]; levels (not shown). La3+ (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.

**[Ca2+] 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 [Ca2+] 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 [Ca2+], that parallels the increase in cell area, most cells had a steady basal level of [Ca2+], 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 [Ca2+], varied substantially between individual cells. The shift in [Ca2+], 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 [Ca2+]. Because illumination of FURA2-loaded cells is eventually toxic, it was not feasible to observe single cells long enough to observe the decline in [Ca2+], that occurred at later times.

**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, 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, IgG, [Ca2+] was observed to show a transient increase similar to cells on FN (Fig. 6 A). This elevation of [Ca2+] 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 [Ca2+]; (not shown), indicating that the effect on [Ca2+] is specific to anti-integrin IgG. Cells on antibodies to the VN receptor, integrin α,β, also showed a transient rise in [Ca2+] (unpublished data). These results suggest that integrins are able to transduce the observed effects on [Ca2+].

As additional controls, the effect of adhesion to anti-ICAM-1 and anti-VCAM-1 on [Ca2+], was examined. Resting human endothelial cells have modest levels of ICAM-1 (~105 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, 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-1\(\alpha\). 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-\(\beta_1\) mab). Treatment with 50 ng/ml IL-1\(\alpha\) 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-1\(\alpha\) 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-\(\beta_1\) integrin mab is specific.

Previous work has shown that clustering \(\beta_1\) integrins with soluble antibodies is sufficient to induce activation of the Na-H antiporter (Schwartz et al., 1991a) and phosphorylation of proteins on tyrosine (Kornberg et al., 1991). In addition, clustering of \(\beta_2\) integrins on leukocytes with soluble antibodies has been shown to induce elevations in [Ca\(^{2+}\)], (Ng-Sikorski et al., 1991; Pardi et al., 1989). The effect on [Ca\(^{2+}\)]\(_i\) of clustering the \(\beta_1\) integrin was therefore examined.

Cells were incubated in the cold with anti-\(\beta_1\), 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 pH\(_i\) using the same antibody (Schwartz et al., 1991a). 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\(^{2+}\)]. (not shown).

We have also attempted to trigger calcium increases by occupying integrins with RGD peptides. Addition of 0.5 mM of the peptide GRGDS, which completely blocks spreading of cells on FN or VN, failed to induce any change in [Ca\(^{2+}\)], 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 pH\(_i\)

To determine whether the elevation of [Ca\(^{2+}\)], during spreading was responsible for the change in pH\(_i\) that occurs when cells spread on FN, EGTA was added to the medium to block the rise in [Ca\(^{2+}\)]. 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 pH\(_i\) 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\(^{2+}\)], measurements confirmed that [Ca\(^{2+}\)], declined slowly during the time course of spreading (not shown). These results demonstrate that the elevation of pH\(_i\) is not mediated by changes in [Ca\(^{2+}\)], in these cells.

Discussion

Our results show that cell spreading on FN or VN is accompanied by an elevation of [Ca\(^{2+}\)], which requires the pres-
Figure 6. [Ca\(^{2+}\)] in cells plated on anti-integrin IgG. (A) Cells were plated on TS2/16 anti-\(\beta_1\) integrin IgG, and [Ca\(^{2+}\)] measured in ~15 cells at each time point. Similar results were obtained in four experiments. (B) [Ca\(^{2+}\)] was measured in cells plated for 60 min on anti-\(\beta_1\) IgG or on BSA, then EGTA was added and [Ca\(^{2+}\)] measured again. Similar results were obtained in three experiments.

Integrins appear to be involved in triggering the elevation of [Ca\(^{2+}\)] in the medium. [Ca\(^{2+}\)] 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 \(\text{La}^{3+}\) supports the notion that calcium channels in the plasma membrane are probably involved.

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Schwartz et al. Adhesion Triggers Elevation of Intracellular Calcium

The function of integrin-mediated changes in [Ca^{2+}], remains unknown, but a role in cell migration is an attractive speculation. [Ca^{2+}], 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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References

Curry, F. E. 1992. Modulation of venular microvessel permeability by calcium influx into endothelial cells. PASEB (Fed. Am. Soc. Exp. Biol.) J. 6:2456-2466.

Donjacour, A. A., and G. R. Cunha. 1991. Stromal regulation of epithelial function. Cancer Treat. Res. 53:335-364.

Dustin, M. L., and T. A. Springer. 1988. Lymphocyte function associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell Biol. 107:321-331.

Godinich, M., M. S. Lapointe, and D. C. Buttle. 1991. Free cytosolic calcium regulation via Na^+-Ca^{2+} exchange in cultured vascular smooth muscle cells. In Sodium-Calcium Exchange. Annals of the New York Academy of Sciences, Vol. 639, M. P. Blaustein, R. Dipolo, and J. P. Reeves, editors. New York: 561-565.

Grzesiak, J. J., G. E. Davis, D. Kirchhofer, and M. D. Pierschbacher. 1992. Regulation of G2B1-mediated fibroblast migration of type I collagen by shifts in the concentrations of extracellular Mg^{2+} and Ca^{2+}. J. Cell Biol. 117:119-117.

Hynes, R. O. 1992. Integrins: versatility, modulation and signaling in cell adhesion. Cell. 69:11-25.

Ingber, D. E., and J. Folkman. 1989. How does extracellular matrix control capillary morphogenesis? Cell. 58:803-805.

Ingber, D. E., D. Prusty, J. F. Frangioni, E. J. Cragoe Jr., C. P. Lechene, and M. A. Schwartz. 1990. Control of intracellular pH and growth by fibronectin in capillary endothelial cells. J. Cell Biol. 110:1803-1811.

Kimmel, M., J. P. Garder, and A. Aviv. 1990. Agonist evoked alkaline shift in the cytosolic pH set point for activation of the Na/H antiport in human platelets. The role of cytosolic calcium and protein kinase C. J. Biol. Chem. 265:21068-21074.

Kornberg, L. J., H. S. Earp, C. E. Turner, C. Prockop, and R. L. Juliano. 1991. Free cytosolic calcium regulation via Na^+-Ca^{2+} exchange in cultured vascular smooth muscle cells. In Sodium-Calcium Exchange. Annals of the New York Academy of Sciences, Vol. 639, M. P. Blaustein, R. Dipolo, and J. P. Reeves, editors. New York: 561-565.

Lakos, Z., B. Somogyi, M. Balazs, J. Matko, and S. Damjanovich. 1990. The effects of transmembrane potential on the dynamic behavior of cell membranes. Biochim. Biophys. Acta. 1023:41-46.

Levin, E. G., and L. Sanetel. 1987. Association of plasmogen activator inhibitor (PAI-1) with the growth substratum and membrane of human endothelial cells. J. Cell Biol. 105:2543-2549.

Manganel, M., and R. J. Turner. 1990. Agonist induced activation of Na+/H+ exchange in parotid acinar cells is dependent on calcium but not protein kinase C. J. Biol. Chem. 265:4284-4289.

Miekka, S. I., K. C. Ingham, and D. Menache. 1982. Rapid methods for isola-

Figure 9. Effect of medium calcium on pH. 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.
Ng-Sikorski, J., R. Andersson, M. Patarroyo, and T. Andersson. 1991. Calcium signaling capacity of the CD11b/CD18 integrin on human neutrophils. Exp. Cell Res. 195:504–508.

Ober, S. S., and A. B. Pardee. 1987. Both protein kinase C and calcium mediate activation of the Na+/H antiporter in Chinese hamster embryo fibroblasts. J. Cell Physiol. 132:311–317.

Pardi, R., T. R. Bender, C. Dettori, E. Giannazza, and E. G. Engelmann. 1989. Heterogeneous distribution and transmembrane signaling properties of lymphocyte function associated antigen (LFA-1) in human lymphocyte subsets. J. Immunol. 143:3157–3166.

Schwartz, M. A., and C. Lechene. 1992. Adhesion is required for protein kinase C dependent activation of the Na+-H antiporter by PDGF. Proc. Natl. Acad. Sci. USA. 89:6138–6141.

Schwartz, M. A., C. Lechene, and D. E. Ingber. 1991a. Insoluble fibronectin activates the Na+-H antiporter by clustering and immobilizing integrin α5β1, independent of cell shape. Proc. Natl. Acad. Sci. USA. 88:7849–7853.

Schwartz, M. A., D. E. Ingber, M. Lawrence, T. A. Springer, and C. Lechene. 1991b. Multiple integrins share the ability to induce elevation of intracellular pH. Exp. Cell Res. 195:533–535.

Schwartz, M. A., G. Both, and C. Lechene. 1989. The effect of cell spreading on cytoplasmic pH in normal and transformed fibroblasts. Proc. Natl. Acad. Sci. USA. 86:4525–4529.

Shimizu, Y., and S. Shaw. 1991. Lymphocyte interactions with extracellular matrix. FASEB (Fed. Am. Soc. Exp. Biol.) J. 5:2292–2299.