Mechanically Induced Calcium Mobilization in Cultured Endothelial Cells Is Dependent on Actin and Phospholipase

Scott L. Diamond, Fred Sachs, Wade J. Sigurdson

Abstract We sought to evaluate the mechanisms by which mechanical perturbation elevates intracellular calcium in endothelial cells. We report that the transient elevation in intracellular calcium in cultured bovine aortic endothelial cells (BAEC) in response to gentle perturbation with the side of a micropipette was not blocked by depolarization (external K+ 130 mmol/L, nifedipine (10 μmol/L), or Bay K 8644 R(+) (10 μmol/L). Thus, voltage-dependent calcium channels were not involved in the response. Also, amiloride (10 μmol/L) and tetraethylammonium (1 mmol/L) had no effect on calcium mobilization, indicating that Na+ and K+ transporters were not involved. Pretreatment of the cells with the phospholipase C and phospholipase A2 inhibitor mannoside (10 μmol/L) for 5 minutes at 37°C completely abolished the calcium response, as did a 10-minute pretreatment with the inhibitor of actin polymerization, cytochalasin B (1 μmol/L). We observed an inhibitory effect of the phospholipase A2 and phospholipase C inhibitor 4-bromophenacyl bromide (10 μmol/L) on the mechanical response of BAEC that was not as potent as that observed with mannoside. To examine the role of arachidonic acid (AA) and subsequent metabolites that may be released after a putatively mechanical activation of phospholipase A2, we exposed BAEC to exogenous AA. We found that continued exposure of BAEC for 5 minutes to 10 mmol/L to 10 μmol/L AA caused no elevation of intracellular calcium. If mechanical stimulation activates phospholipase A2, the liberated AA and subsequent metabolites do not appear to have much effect on BAEC intracellular calcium. Because extracellular calcium was required for the elevation of intracellular calcium, we suggest that mechanical deformation activates a plasma membrane ion channel permeable to extracellular calcium, and provides an amount of calcium sufficient to trigger release of internal calcium stores. Actin filaments may be required or involved in either the transfer of forces to the channel or in the subsequent activation of Ca2+-dependent phospholipases. (Arterioscler Thromb. 1994;14:2000-2006.)

Key Words • endothelium • mechanotransduction • biomechanics • intracellular calcium • phospholipase

As the interface between the vessel wall and flowing blood, the endothelium must function in a complex fluid mechanical and biomechanical environment. Endothelial cell adjustments to physical stimuli may have roles in various types of vessel physiology and pathology. The fastest endothelial responses to chemical stimuli occur on a time scale of milliseconds to seconds and include ion channel regulation and receptor-mediated signal transduction. These pathways are fairly well characterized in endothelial cells (for review, see Reference 1). The endothelial response to the onset of steady laminar fluid shear stress is also rapid. Flow-activated hyperpolarization of bovine aortic endothelial cells (BAEC) by means of the activation of a K+-selective whole cell current requires about 10 seconds and is half maximal at a shear stress of 0.07 N/m2 (0.7 dyne/cm²). For cultured rat aorta endothelial cells subjected to flow, the net intracellular acidification brought about by activation of both Na+-independent Cl-/HCO3- exchangers and Na+/H+ exchangers requires about 30 seconds to reach a full response and is half maximal at a shear stress of about 0.1 N/m².

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From the Departments of Chemical Engineering (S.L.D.) and Biophysical Sciences (F.S., W.J.S.), The State University of New York at Buffalo, New York.
Correspondence to Scott L. Diamond, PhD, Bioengineering Laboratory, Department of Chemical Engineering, 907 Furnas Hall, Buffalo, NY 14260.
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The endothelial response to the onset of flow includes a large burst of prostacyclin (PGI2),4 and nitric oxide production5 and generation of inositol trisphosphate (IP3),6 as measured in human umbilical vein endothelial cells and vessel preparations. The generation of PGI2, nitric oxide, and IP3 is indicative of an elevation of intracellular calcium. However, experimental demonstration of shear-induced calcium mobilization (in the absence of exogenous ATP) has been variable both in cultured endothelial cells (observed in References 7 through 9; not observed in References 3 and 10 through 15) and in vessel preparations (observed in Reference 16; not observed in Reference 17). In contrast to this experimental variability of the shear stress response, the endothelial response to mechanical deformation has repeatedly been shown to include a rapid and large elevation of intracellular calcium,12,18,21 as measured by a variety of calcium-sensitive fluorescent dyes. We and others have shown that the mobilization of intracellular calcium requires some extracellular calcium.15,20,21 This calcium activation due to cellular distortion or deformation occurs in lung epithelial cells,22 heart cells,24 mast cells,25 glial cells,26 and kidney cells.27

Along with the elevation of calcium in the mechanically stimulated endothelial cell, a wave of elevated calcium similar to the calcium spread in other mechanically activated cell types such as glial28 and lung epithelial cells23 moves outward in neighboring cells from the site of stimulation.15,19,20 In the endothelial cell stimulated by mechanical perturbation, the calcium
The micropipette was controlled by means of a micromanipulator to perform single frames using a Macintosh IIci with a Quickcapture analyser. The intensity of a 10 x 10 pixel region was averaged to analyze the temperature, recorded in real time on S-VHS videotape, and corresponding to the focal plane of the image seen on differential interference contrast (DIC) microscopy. Epifluorescence and DIC observations of endothelial cells were conducted on a Zeiss Axiovert (40 x planapochromat, NA 1.4) mounted with a Zeiss AxioCam II and an intensified (Videoscope) charge-coupled device (Dage MTI) camera. All experiments were conducted at room temperature, recorded in real time on S-VHS videotape, and analyzed by averaging the intensity of a 10 x 10 pixel region of individual frames using a Macintosh IIci with a Quickcapture frame grabber (Data Translation) and NIH Image 1.49. No background subtraction, frame averaging, or contrast enhancement was carried out on the video signal used for analysis of fluorescence intensity.

Methods

Reagents

All pharmacological agents except for manoolide (Calbiochem) and cytochalasin B (Sigma) were obtained from Research Biochemicals Inc. Inhibitors were dissolved in water, dimethylsulfoxide, or ethanol as recommended by the manufacturers. These stock solutions were used at 1:100 to 1:1000 dilution in HEPES buffered saline (HBS) ([mmol/L] NaCl 140, KCl 5, MgCl_2 1, CaCl_2 1, and HEPES 10 and 0.1% [wt/vol] bovine serum albumin). The concentration of NaCl was reduced to 10 mmol/L for the high-K^+ solution (K^+, 130 mmol/L) experiments.

Cell Culture and Dye Loading

BAEC were used between passages 6 and 10. These cells were grown on NaOH-treated 25-mm coverslips in 10% new-born calf serum (Gibco BRL) in DMEM (Dulbecco’s modified Eagle’s medium) (Gibco BRL) supplemented with penicillin and streptomycin as previously described using standard methodologies. Monolayers were loaded for 30 minutes at room temperature in 4 μmol/L Flu-o-3/AM (Molecular Probes) dispersed with 0.2% pluronic F-127, washed, and incubated for an additional 20 minutes in HBS to allow hydrolysis of the dye. In all experiments, the cells displayed a diffuse calcium-sensitive fluorescence as previously seen, indicating absence of dye localization into organelles.

Epifluorescence Video Microscopy and Analysis

Endothelial cells were stimulated by gentle movement of the side (not tip) of a fire-polished micropipette bent to achieve a parallel orientation to the cell as previously described. The micropipette was controlled by means of a micromanipulator with a calibrated z-axis position digital readout (Burleigh). The regimen for cell stimulation involved a 1- to 2-μm deformation of endothelial cells, which were typically 5 to 8 μm thick as measured by a second position readout corresponding to the focal plane of the image seen on differential interference contrast (DIC) microscopy. Epifluorescence and DIC observations of endothelial cells were conducted on a Zeiss Axiovert (40 x planapochromat, NA 1.4) mounted with an intensified (Videoscope) charge-coupled device (Dage MTI) camera. All experiments were conducted at room temperature, recorded in real time on S-VHS videotape, and analyzed by averaging the intensity of a 10 x 10 pixel region of individual frames using a Macintosh IIci with a Quickcapture frame grabber (Data Translation) and NIH Image 1.49. No background subtraction, frame averaging, or contrast enhancement was carried out on the video signal used for analysis of fluorescence intensity.

Results

In endothelial cultures that respond to mechanical prodding, the probability of response (calcium mobilization) was very high (>95% of cells tested responded to the first pipette prodding). Before testing the effect of depolarization on the calcium response of stimulated cells, we verified that two of two cells tested responded under our standard regimen of mechanical perturbation of 1- to 2-μm deformations in the presence of HBS (Fig 1). After this demonstration, we exchanged HBS with high-K^+ solution to cause no change in the intracellular calcium concentration. This finding is distinct from observations of depolarization of heart cells, which causes large mobilization of calcium through voltage-dependent Ca^2+ channels (data not shown). Unlike in heart cells, in BAEC the increases in intracellular calcium were identical to the control response in three of three cells tested upon mechanical activation in the presence of high levels of K^+.

We examined the effects of the well-characterized calcium channel antagonists nifedipine (10 μmol/L) and...
Bay K 8644 R(+) (10 μmol/L). Neither agent blocked the rise in intracellular Ca\(^{2+}\) that was caused by mechanical perturbation of BAEC (Fig 2). This finding is consistent with that in the previous experiment in which high-K\(^+\) media had no effect, indicating that mechanical perturbation does not cause depolarization that leads to increases in intracellular calcium.

Endothelial cells in culture have several well-characterized ion transporters that include the calcium-activated K\(^+\) channel (K\(_{Ca}\)) and the Na\(^+\)/H\(^+\) exchanger. Inhibition of K\(_{Ca}\) with tetrathylinammonium ion (TEA, 1 mmol/L) had no effect on the ability of BAEC to respond to mechanical activation (Fig 3). We found that TEA caused a very slight increase in intracellular calcium that was not reliably above the noise level of our fluorescence intensity signal. Similarly, amiloride had little effect on the increases of intracellular calcium in cells that were mechanically prodded with a micropipette. Amiloride caused no change in intracellular calcium upon its addition to the cells.

We observed some heterogeneity in the kinetics of the return to baseline of the calcium signal after mechanical stimulation. This may be due to variations from cell to cell, variations in the amount of force applied to each cell tested, or the effect of the pharmacological agent on calcium homeostasis.

Preincubation of BAEC with cytochalasin B (1 mmol/L) for 10 minutes led to an inhibition of Ca\(^{2+}\) increase in mechanically stimulated endothelial cells (Fig 4). Large crushing mechanical deformations of the cell caused increases in intracellular calcium (Fig 4B). In this regimen of extreme cellular deformation, the micropipette came directly into the focal plane of the epifluorescence observation and could be precisely visualized. We found that this extensive cellular deformation could bring about an elevation in intracellular Ca\(^{2+}\). This experimental regimen is in excess of our normal cellular deformation that includes membrane blebbing.\(^{13}\) We found that endothelial cells were responsive to gentle mechanical prodding during the first 5 minutes of exposure to cytochalasin B, indicating that a few minutes were required for full disruption of the actin cytoskeleton. Pretreatment of BAEC for 20 minutes with cytochalasin B had no effect on the responsiveness of the cells to 10 μmol/L ATP, indicating that the purinergic receptor and signal transmission pathway were still intact.

Manoalide is a time- and temperature-dependent inhibitor of phospholipases.\(^{29}\) Generally, the use of manoalide requires preincubation for 10 minutes at 37°C to achieve full inhibitory action. In our experiments, Fluo-3/AM-loaded cells were incubated in 10 μmol/L manoalide in a CO\(_2\) incubator at 37°C. Manoalide used at a lower concentration without preincubation (1 μmol/L at room temperature) did not block the response of the endothelial cell to our standard regimen of cellular perturbation. This result is consistent with previous observations by Wheeler et al\(^{29}\) that the inhibitory action of manoalide is time- and temperature-dependent in GH3 cells. After a 10-minute incubation with manoalide at 10 μmol/L at 37°C, BAEC were unresponsive to the mechanical stimulation (1-μm deformation) that would normally increase intracellular Ca\(^{2+}\) (Fig 5). By continuing the downward movement of the micropipette beyond the extent at which a normal cell response was expected but not seen, we could essentially crush the endothelial cell (as in Fig 4B) and evoke a response in the presence of manoalide, as was seen with cells in the presence of cytochalasin B.

We tested the effect of 4-bromophenacyl bromide (pBPB) (10 μmol/L) on the mechanically induced calcium response. pBPB is a widely used phospholipase A\(_2\) inhibitor, but it is reported to also inhibit phospholipase C.\(^{30}\) We observed an inhibitory effect of pBPB on the mechanical response of BAEC (Fig 6), but the inhibition was not as potent as that observed with manoalide.

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**Fig 2.** Tracings indicating that calcium antagonists nifedipine (10 μmol/L) and Bay K 8644 R(+) (10 μmol/L) had no effect on the mechanically induced calcium mobilization of bovine aortic endothelial cells. Two of two cells tested displayed normal responses in the presence of each of these voltage-dependent calcium channel blockers. F/F\(_B\) indicates ratio of fluorescence after mechanical stimulation to baseline fluorescence.

**Fig 3.** Tracings demonstrating that the K\(^+\) channel blocker tetrathylinammonium ion (TEA, 1 mmol/L) and the Na\(^+\) exchanger inhibitor amiloride (10 μmol/L) had no effect on the mechanically induced calcium mobilization of bovine aortic endothelial cells. F/F\(_B\) indicates ratio of fluorescence after mechanical stimulation to baseline fluorescence.
FIG 4. Left, Tracings showing that cytochalasin B inhibited the calcium response of bovine endothelial cells in mechanically activated endothelial cells, indicating the requirement for actin. Two of two cells in the monolayer (left, top two recordings) were tested for display of control response. The monolayer was incubated in 10 \( \mu \)mol/L cytochalasin B for 10 minutes, after which cells became unresponsive to mechanical stimulation (left, bottom five recordings). The first arrow indicates time of contact of the pipette with the cell. Further movement of the pipette through the cell toward the glass substrate caused severe cellular deformation and eventually led to calcium increase, as indicated by the second arrow in each tracing. Right, Travel of the pipette deep into the cell and into the focal plane led to a clear visualization of the micropipette, which was not apparent when cells were activated under control conditions. F/F_0 indicates ratio of fluorescence after mechanical stimulation to baseline fluorescence.

Only a third of the cells stimulated in the presence of pBPB displayed a calcium response upon the first mechanical perturbation, but none of the cells tested in the presence of pBPB responded a second time to prodding. We found that pBPB blocked the response of BAEC to 10 \( \mu \)mol/L ATP, which is indicative of PLC inhibition.

To test the role of AA and subsequent metabolites that may be released after a putatively mechanical activation of phospholipase A_2, we exposed BAEC to exogenous AA. We found that continued exposure of BAEC (passage 6) for 5 minutes to 10 nmol/L, 100 nmol/L, 1 \( \mu \)mol/L, and 10 \( \mu \)mol/L AA caused no elevation of intracellular calcium. Other researchers have also seen that AA had no effect on intracellular calcium levels in BAEC (W.J. Schilling, personal communication, 1994). Also, AA is not an inducer of IP_3 in endothelial cells. If mechanical stimulation activates phospholipase A_2, the liberated AA and subsequent metabolites do not appear to have much effect on intracellular calcium in BAEC.

As a control for the physicochemical effect of the glass pipette surface in contact with the membrane of the cell, we created mechanical deformation in cells using impinging flows from pressurized micropipettes. In this control experiment, an impinging flow (3-\( \mu \)m-orifice pipette held 3 \( \mu \)m above and normal to the cell) was created with a pipette backpressure of 250 mm Hg. This impinging flow caused a slight dimple in an endothelial cell that led to calcium mobilization. Thus, the chemical interaction of a glass material in close contact with the cell membrane was not required for calcium mobilization in cells undergoing mechanical deformation.

FIG 5. Tracings showing that mannoalide inhibits the calcium response of bovine endothelial cells in mechanically activated endothelial cells, indicating the requirement for phospholipase C or phospholipase A_2. Eight of eight cells failed to respond to deformations that would normally activate endothelial cells. The monolayer was incubated in 10 \( \mu \)mol/L mannoalide at 37°C for 10 minutes, after which cells became unresponsive to mechanical stimulation. The inverted triangle indicates point of contact of the pipette with the cell. Further movement of the pipette through the cell toward the glass substrate caused severe cellular deformation and eventually led to calcium increase, as indicated by the second arrow in each tracing. F/F_0 indicates ratio of fluorescence after mechanical stimulation to baseline fluorescence.
Fig 6. Bar graph of the partial inhibition by the phospholipase A2 and phospholipase C inhibitor, 4-bromophenacyl bromide (10 μmol/L), of the mechanically induced calcium response. Control cells displayed a positive response to first deformation (shaded bars) in 11 of 12 cells tested and a positive response to second deformation (open bars) in 6 of 7 cells tested. Response rate (number of responding cells/total cell number tested) decreased substantially in the presence of pBPB.

Discussion

We found that the rapid elevation of intracellular calcium in mechanically activated BAEC is dependent on an intact actin cytoskeleton and can be blocked by inhibition of phospholipase C and phospholipase A2. Voltage-dependent Ca2+ channels, KCa, and Na+ exchangers are not required for the response. Other researchers have documented that depolarization of endothelial cells by itself does not cause an increase in intracellular calcium.12 In our earlier work13 we found that the response is dependent on the presence of extracellular Ca2+. Because manoalide, an inhibitor of phospholipases, could block the response, the calcium detected by Fluo-3 was the calcium released by the cell and was not from the influx of extracellular calcium. It remains possible that manoalide inhibits the mechanically sensitive channel.

Manoalide isolated from the sponge Luffariella variabilis is a potent inhibitor of several forms of phospholipase A2 (IC50, 0.05 to 2 μmol/L) and phosphatidylinositol-specific phospholipase C (IC50, 1.5 μmol/L) from mammalian sources, as well as voltage-dependent Ca2+ channels (IC50, 1.0 μmol/L).14 Given the lack of effect of depolarization, nifedipine, and Bay K 8644 R(+) in blocking the increase in calcium in mechanically activated endothelial cells, the inhibitory effect of manoalide does appear to involve its action on lipid metabolism. In fact, voltage-dependent Ca2+ channels in cultured endothelial cells have been unusually difficult to find.1 Because manoalide and pBPB inhibit phospholipase A2 and phospholipase C, it was not possible to identify which enzyme was required for the mechanical response. Although free AA generated by phospholipase A2 can act as a modulator of signal transduction in some cell types,31 phospholipase C is the more likely participant in amplifying the calcium mobilization in endothelial cells by means of an IP3 pathway. We have found that AA has little effect on BAEC intracellular calcium. The recent observation that intracellular heparin acts as an inhibitor of IP3-mediated Ca2+ release in mechanically activated lung epithelial cells23 is consistent with our observations with manoalide and our hypothesis that phospholipase C is involved in the endothelial response. Also, it has been shown that IP3 levels increase in endothelial cells subjected to cyclic stretch.34

Cytochalasin B is a potent inhibitor of actin polymerization. Nawroth et al15 found that cytochalasin B, cytochalasin D, and colchicine, either by a direct effect on the cytoskeleton or by an unknown mechanism, can inhibit PGI2 production over several hours in endothelial cells stimulated with phorbol ester or endotoxin. Alteration of the cytoskeleton had little effect on cyclooxygenase levels because A23187 stimulation of PGI2 production was unaffected by the cytoskeletal inhibitors. This would suggest that the integrity and normal functionality of signal transduction (upstream of cyclooxygenase) in the plasmalemma require correct architecture of the membrane-cytoskeletal coupling. In our measurements, which occurred in a time frame much shorter than in the study by Nawroth et al, cytochalasin B caused a complete blockade of calcium response in mechanical stimulation of endothelial cells. We have shown that short-term treatment of BAEC with cytochalasin B does not disrupt receptor-mediated signaling. It is possible, given our findings with manoalide, that depolymerization of actin by cytochalasin B leads to loss of functionality of phospholipase-mediated signaling (independent of the forces applied to the membrane), loss of actin-dependent concentration of macroscopic stresses directed at the molecular-level mechanosensor of the membrane, loss of functionality of the molecular-level mechanosensor independent of stress concentration by actin, or all three phenomena. Alternatively, actin and actin-associated enzymes may be the source of second messengers upon cellular deformation, but this hypothesis is not consistent with the requirement of extracellular calcium for the response.15,20

In light of these findings, we propose the following model of the molecular processes by which endothelial cells respond to mechanical stimulation (Fig 7). Exposure of the endothelial cell to mechanical deformation leads to the actin-dependent opening of a Ca2+-permeable channel that is not inhibited by nifedipine, amiloride, or TEA. This calcium near the inner plasmalemma leads to the activation of phospholipase C, which amplifies the calcium response through IP3-mediated release of intracellular calcium. Within about a second, the calcium in the cell reaches peak levels and causes a myriad of cellular events. Within about a minute after mechanical stimulation, the cell returns to resting con-
tions, and calcium is sequestered back into internal stores or pumped out of the cell.

Acidification of endothelial cells by fluid shear stress is due to the net alkali extrusion by the Cl⁻/HCO₃⁻ exchanger in excess of the shear stress activation of the Na⁺/H⁺ exchanger, the acid extruder.³ Mechanical deformation may cause the direct activation of the Na⁺/H⁺ exchanger (with transient alkalinization in the absence of external HCO₃⁻), followed by Na⁺/Ca²⁺ exchange and subsequent amplification of the calcium signal by means of calcium-induced calcium release or Ca²⁺-activated signaling. Such a pathway has been observed in cardiac myocytes.³⁶ However, the absence of an effect of amiloride on the Ca²⁺ mobilization observed in mechanically activated endothelial cells would indicate that Na⁺/H⁺ exchange is not required for the response. Still, the Na⁺/H⁺ exchanger may be activated directly by mechanical deformation, in a manner analogous to shear stress activation,³ but the transient alkalinization or Na⁺/Ca²⁺ exchange of this putative process is not responsible for the observed changes in Ca²⁺.

In addition, ATP can activate the amiloride-sensitive Na⁺/H⁺ exchanger in cultured BAEC, and this activation requires extracellular calcium.³⁷ A23187 can also activate the Na⁺/H⁺ exchanger in cultured BAEC, suggesting that elevated levels of intracellular calcium can lead to cellular alkalinization. From the work of Kitazono et al,³⁷ we would expect that the rapid rise in intracellular calcium observed by mechanical perturbation of the cell would activate this channel, leading to Na⁺-dependent intracellular alkalinization. Conversely, elevation of intracellular Ca²⁺ in smooth muscle cells has been associated with ATP-dependent H⁺ influx and acidification, possibly through the action of the plasma membrane Ca²⁺-ATPase, not the Ca²⁺/H⁺ exchanger.³⁸ Without direct intracellular pH (pHᵢ) measurements in the presence and absence of extracellular HCO₃⁻, the net effect of mechanically induced calcium increases on pHᵢ in endothelial cells is difficult to predict.

The endothelial response to mechanical forces on short and long time scales depends on the precise quantitative makeup of that force. The shear stress generated by blood flow is distributed somewhat uniformly across the cell plasmalemma to the load-bearing elements of the cytoskeleton and adhesion plaques. Substrate stretching and micropipette prodding are similar in that each causes macroscopic deformation of cellular structures near a stretched plasmalemma. Hypotonic swelling of cells creates tensions in the stretched membrane as well as macroscopic deformation of cellular structures throughout the cell, in addition to changes in intracellular concentrations.

The micropipette prodding assay is a model system to study the effect of mechanical deformation on endothelial function at the single-cell level. In vivo, endothelial cells can experience macroscopic deformation in various physiological and pathological instances. Postsurgery deep vein thrombosis is associated with venous distention and perturbation of endothelial function.³⁹ Conversely, during vasoconstriction that accompanies chronic low flow states, the internal elastic lamina and endothelium display marked waviness that requires large changes in endothelial conformation.⁴⁰ In addition, the pulsatile blood flow in arteries causes strains of 1% to 10 % in the intima. Finally, cardiovascular surgery and catheter procedures impose direct mechanical forces on the endothelium in the lyric and sublytic range.

Given the impact of intracellular calcium and pHᵢ on the timing of mitogenesis, mechanical stress may initiate endothelial mitogenesis by means of calcium increases and putative changes in pHᵢ analogous to the changes seen during growth factor stimulation of endothelial cells. In the in vitro substrate stretching assays that cause mechanical deformation of endothelial cells, elevations in intracellular calcium⁴¹ and increased mitogenesis⁴¹ are observed. Mitotic endothelial cells are particularly leaky with respect to macromolecule transport,⁴² and these mitotic events tend to occur in regions of complex time-dependent flows as well as in regions subjected to mechanical strain. Our observations may offer some insight into the initial mechanisms by which specific geometric locations subject to high strains in the cardiovascular system are associated with elevations of mitosis or other Ca²⁺-dependent alterations of function such as permeability.⁵³

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S L Diamond, F Sachs and W J Sigurdson

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