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 tetrathylammonium (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 mannoalide (10 μmol/L) for 10 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 mannoalide. 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 plasmalemma 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.

© 1994 American Heart Association, Inc.

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+/H+ and Na+/K+ exchanging and Na+/H+ exchanging requires about 30 seconds to reach a full response and is half maximal at a shear stress of about 0.1 N/m2.

Received April 6, 1994; accepted September 14, 1994.
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.
© 1994 American Heart Association, Inc.
elevation moves rapidly outward from the point of stimulation toward the periphery of the cell in 300 to 500 milliseconds, yet the movement is slower than diffusion. Within a single endothelial cell, the calcium wave velocity was found to be about 50 μm/s. Neighboring cells of the mechanically stimulated cell become activated about 1 second after full response is seen in the stimulated cell. Presumably, cell-to-cell communication in touching cells occurs through gap junctional transport of second messengers such as Ca^{2+} or IP_{3}.^{23}

In the present study, we sought to characterize the calcium response of mechanically activated endothelial cells in terms of requisite pathways. Pharmacological inhibitors can serve as tools to explore these phenomena. We used blockers of Ca^{2+}, Na^{+}, and K^{+} transporters; inhibitors of signal transduction; and inhibitors of cytoskeletal function. We found that the calcium mobilization in endothelial cells in response to mechanical perturbation is phospholipase dependent and actin dependent, but is independent of voltage-dependent calcium channels, K^{+} channels, and amiloride-sensitive Na^{+} transporters.

**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^{+} (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% newborn 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 Fluo-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 Axiosvert (40× 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 10×10 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 did not cause an increase in intracellular calcium. Three of three cells tested in the presence of high K^{+} displayed a regulated calcium response to mechanical stimulation when tested under conditions of depolarization (bottom three recordings). Arrow indicates time of contact of pipette with cell. F/F indicates ratio of fluorescence after mechanical stimulation to baseline fluorescence.

![Fig 1. Tracings of depolarization. High-K^+ solution (130 mmol/L) had no effect on the mechanically induced calcium mobilization of bovine aortic endothelial cells. Two of two cells tested were responsive to mechanical stimulation under control conditions in normal HEPES buffered saline (HBS), indicating that cells of the monolayer were responsive (top two recordings). Exchange of HBS with high-K^+ solution did not cause an increase in intracellular calcium. Three of three cells tested in the presence of high K^+ displayed a regulated calcium response to mechanical stimulation when tested under conditions of depolarization (bottom three recordings). Arrow indicates time of contact of pipette with cell. F/F indicates ratio of fluorescence after mechanical stimulation to baseline fluorescence.](image-url)
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\(_c\)) and the Na\(^+\)/H\(^+\) exchanger. Inhibition of K\(_c\) with tetraethylammonium 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 μmol/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 responses in the presence of each of these voltage-dependent calcium channel blockers. F/F\(_0\) indicates ratio of fluorescence after mechanical stimulation to baseline fluorescence.

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.
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 μmol/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 A₂, 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 μmol/L, and 10 μmol/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 IP₃ in endothelial cells.³¹ If mechanical stimulation activates phospholipase A₂, 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-μm-orifice pipette held 3 μm 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.
is consis-tematically activated lung epithelial cells calcium. The recent observation that intracellular hep-

hypothesis that phospholipase C is involved in the release in arin acts as an inhibitor of IP

found that AA has little effect on BAEC intracellular response. Although free AA generated by phospholi-

zymes may be the source of second messengers upon pholipase-mediated signaling (independent of the forces

tation was unaffected by the cytoskeletal inhibitors. This finding with manoalide, that depolymerization of actin

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-A 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, 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 cells 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.

Cytochalasin B is a potent inhibitor of actin polymerization. Nawroth et al 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 endothxin. 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.

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-
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 exchanger.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 (pH₇) measurements in the presence and absence of extracellular HCO₃⁻, the net effect of mechanically induced calcium increases on pH₇ 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 lytic and sublytic range.

Given the impact of intracellular calcium and pH₇ on the timing of mitogenesis, mechanical stress may initiate endothelial mitogenesis by means of calcium increases and putative changes in pH₇ 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

Acknowledgments

This research was supported by NSF grant BCS9211197 (S.L.D.), NIH grant HL47486 (S.L.D.), and US Army Research Grant DAA10389K0064 (F.S.). Dr Diamond is a recipient of the National Science Foundation National Young Investigator award.

References


17. Curry FE, He P. Shear stress does not increase cytoplasmic calcium concentration in individually perfused microvessels. Presented at the annual fall meeting of the Biomedical Engineering Society; October 17, 1992; Salt Lake City, Utah.


Mechanically induced calcium mobilization in cultured endothelial cells is dependent on actin and phospholipase.
S L Diamond, F Sachs and W J Sigurdson

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/14/12/2000

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/