Endothelial Cell Cytosolic Free Calcium Regulates Neutrophil Migration across Monolayers of Endothelial Cells

Ada J. Huang,* John E. Manning†, Tania M. Bandak‡, Michelle C. Ratau‡, Katharine R. Hanser‡, and Samuel C. Silverstein~
Departments of *Medicine and †Physiology and Cellular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Abstract. Polymorphonuclear leukocytes (PMN) traverse an endothelial cell (EC) barrier by crawling between neighboring EC. Whether EC regulate the integrity of their intercellular adhesive and junctional contacts in response to chemotaxing PMN is unresolved. EC respond to the binding of soluble mediators such as histamine by increasing their cytosolic free calcium concentration ([Ca++]t) (Rotrosen, D., and J.I. Gallin. 1986. J. Cell Biol. 103:2379-2387) and undergoing shape changes (Majno, G., S. M. Shea, and M. Leventhal. 1969. J. Cell Biol. 42:617-672). Substances such as leukotriene C4 (LTC4) and thrombin, which increased the permeability of EC monolayers to ions, as measured by the electrical resistance of the monolayers, transiently increased EC [Ca++]t. To determine whether chemotaxing PMN cause similar changes in EC [Ca++]t, human umbilical vein endothelial cells (HUVEC) maintained as monolayers were loaded with fura-2. [Ca++]t was measured in single EC during PMN adhesion to and migration across these monolayers. PMN-EC adhesion and transendothelial PMN migration in response to formyl-methionyl-leucyl-phenylalanine (fMLP) as well as to interleukin 1 (IL-1) treated EC induced a transient increase in EC [Ca++]t, which temporally corresponded with the time course of PMN-EC interactions. When EC [Ca++]t was clamped at resting levels with a cell permeant calcium buffer, PMN migration across EC monolayers and PMN induced changes in EC monolayer permeability were inhibited. However, clamping of EC [Ca++]t did not inhibit PMN-EC adhesion. These studies provide evidence that EC respond to stimulated PMN by increasing their [Ca++]t, and that this increase in [Ca++]t causes an increase in EC monolayer permeability. Such [Ca++]t increases are required for PMN transit across an EC barrier. We suggest EC [Ca++]t, regulates transendothelial migration of PMN by participating in a signal cascade which stimulates EC to open their intercellular junctions to allow transendothelial passage of leukocytes.

A critical and precisely regulated step in acute inflammation is the movement of polymorphonuclear leukocytes (PMN) across the vascular endothelium. This process can be initiated by PMN in response to soluble chemoattractants or by endothelial cells (EC) in response to cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (8, 29). Cell surface molecules including the CD11/CD18 complex of proteins on the PMN surface (12) and intercellular adhesion molecule 1 (ICAM 1) (4), endothelial leukocyte adhesion molecule 1 (ELAM 1) (2), and platelet activating factor (35) on the EC surface have been shown to mediate the adhesion of PMN to EC, but the mechanism(s) by which junctions between EC open during PMN emigration from the blood remain unresolved.

Monolayers of cultured EC respond to soluble mediators such as histamine by increasing their permeability to water and macromolecules. Such an EC response requires the soluble mediator to bind to a corresponding receptor on the EC surface. Binding of histamine to histamine receptors on EC results in an increase in EC cytosolic free calcium concentration ([Ca++]t), assembly of actin filaments, and an increase in transendothelial albumin flux in vitro (30). Morphologic studies in vivo demonstrate that changes in EC shape and opening of the interendothelial cell junctions accompany this increase in vascular permeability to fluid and macromolecules (26). Similar changes in EC shape accompany PMN adhesion to and migration across an endothelium.

Address correspondence to Dr. Huang, College of P & S, Columbia University, Department of Physiology, 630 West 168th Street, New York, NY 10032.

1. Abbreviations used in this paper: [Ca++]t, cytosolic free calcium concentration; EC, endothelial cell; ELAM 1, endothelial leukocyte adhesion molecule 1; fMLP, formyl-methionyl-leucyl-phenylalanine; HBS, Hepes buffered saline; HIFBS, heat inactivated fetal bovine serum; HUVEC, human umbilical vein endothelial cells; ICAM 1, intercellular adhesion molecule 1; IL-1, interleukin 1; LTC4, leukotriene C4; M199/FBS, M199 medium containing 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 µg/ml amphotericin B; PMN, polymorphonuclear leukocytes.
(28, 9). Therefore, we hypothesized that PMN-EC interactions initiate intracellular signals similar to those which are initiated when histamine binds to its receptors on EC, namely the generation of EC [Ca++] transients.

To examine whether PMN interactions with EC induce a change in EC [Ca++], we developed a method for measuring [Ca++], in single EC maintained in intact monolayers. We report here that PMN adhesion to and/or migration across an EC monolayer induces a transient increase in EC [Ca++], and that inhibition of this rise in [Ca++] inhibits both PMN-initiated increases in EC monolayer permeability and transendothelial migration of PMN.

Materials and Methods

Materials
Histamine and thrombin were obtained from Sigma Chemical Co. (St. Louis, MO) and leukotriene C4 (LTC4) from Upjohn (Kalamazoo, MI).

Cell Isolation and Culture
Human umbilical vein endothelial cells (HUVEC) were isolated, grown to confluence in gelatin coated 60-mm plastic tissue culture dishes (Corning Glass Works, Corning, NY), removed from the plastic substrate with 0.125% trypsin and 1 mM EDTA in Ca++- and Mg++-free PBS, and re-plated on the stromal surface of human amnion tissue stretched across Teflon rings as previously described (17). HUVEC were maintained in M199 medium (Gibco Laboratories, Grand Island, NY) containing 20% FBS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 #g/ml streptomycin, and 2 #g/ml amphotericin B (M199/FBS). HUVEC cultures on amnion in 24-well plates were moved between 2 and were used from 7-12 d after plating. Previous studies demonstrated that such cultures exhibit maximum transendothelial electrical resistance (6-12 #cm²) during this period (17).

Human Umbilical Vein Endothelial Cell Monolayer Permeability
The permeability of HUVEC monolayers to ions was assessed by measuring transendothelial electrical resistance as previously described (17). Briefly, HUVEC monolayers on amnion in Teflon rings were mounted in a lucite chamber to form a two compartment system with the EC on amnion forming a barrier between the two compartments. Two pairs of Ag/AgCl electrodes (WPI, Inc, New Haven, CT) and 0.9% NaCI bridges were used. DC current was passed through one pair of electrodes in Ohm's law (V = IR). Background resistance of the amnion and medium or background resistance was calculated with the second pair of electrodes and recorded. Resistance of the HUVEC on amnion was measured after each addition. The apical and basal surfaces of a HUVEC monolayer cultured on amnion were incubated with 0.5 and 1.0 ml respec-
tively of this fura-2 AM containing solution for 30 min at 18°C to load the HUVEC with the calcium indicator dye, fura-2. The monolayers were then washed three times with PBS and incubated in PBS containing 2% FBS or BSA (5 mg/ml) for an additional 15 min at 18°C to allow complete hydrolysis of intracellular fura-2 AM to its free acid (calcium sensitive) form. In experiments in which HUVEC [Ca++] were clamped, HUVEC monolayers were incubated with HBS containing FBS (2%), Pluronic (0.02%), and the cell permeant calcium buffer, MAPTAM, (200 μM) (Cistron Technology, Pinebrook, N J) for 4 h at 37°C prior to adding PMN, where indicated. PMN and HUVEC were incubated together for the specified times (usually for 15 min) during which time HUVEC [Ca++] was monitored as described below. Nonadherent PMN were removed by aspiration and HUVEC monolayers were fixed with 10% formalin. PMN migration was quantified as previously described (17). Briefly, cultures were re-moved from Teflon rings and stained with tetrachrome stain (Harleco, Gibbstown, NJ). Stained cultures were mounted on slides and the number of PMN that had migrated was visually assessed by light microscopy.

Cytosolic Free Calcium Measurements
Fura-2 AM (5 μM) (Molecular Probes, Inc., Eugene, OR), Pluronic (0.02%), and the cell permeant calcium buffer, MAPTAM, (200 μM) (Cubiochem Corp., San Diego, CA) were removed by aspiration and HUVEC monolayers were fixed with 10% formalin. PMN migration was quantified as previously described (17). Briefly, cultures were re-moved from Teflon rings and stained with tetrachrome stain (Harleco, Gibbstown, NJ). Stained cultures were mounted on slides and the number of PMN that had migrated was visually assessed by light microscopy.

Standardization of Calcium Measurements

[Ca++] was calculated from ratios of fura-2 fluorescence at two excitation wavelengths as described by Grynkiewicz (11). The Kp of fura-2 for Ca++ at 37°C based on measurements made at 340 and 380 nm with this photome-
tyr system was 213 μM which is in excellent agreement with the value of 224 μM reported by Grynkiewicz (11). Due to the relative lack of light trans-
Figure 1. Schematic drawing of apparatus for making [Ca++]i measurements in single HUVEC maintained in intact monolayers. HUVEC on amnion were loaded with fura-2 and mounted in a water jacketed chamber on the stage of an upright fluorescence microscope which was equipped with a photomultiplier tube. Excitation wavelength was determined by a filter placed between the light source and HUVEC and emission wavelength was determined by a filter placed between HUVEC and the photomultiplier tube. A pinhole stop positioned between the HUVEC and the photomultiplier tube determined the size of the field in which fura-2 fluorescence was measured. Soluble agonists or PMN were added to the compartment above the buffer or a chemoattractant were placed in the compartment below HUVEC monolayers.

mission at 340 nm through the glass optical system of the microscope used, quantitative measurements of fura-2 fluorescence were performed at excitation wavelengths of 350 and 380 nm; the Kd of fura-2 for Ca++ at 37°C based on measurements made at these wavelengths was 179 nM. Therefore 179 nM was used to calculate [Ca++]i from ratios of fura-2 fluorescence for all experiments currently reported.

Results

Human Umbilical Vein Endothelial Cells Consistently Load and Hydrolyze the Calcium Indicator Dye, Fura-2

The fluorescence signal intensity generated by single HUVEC loaded with fura-2 was sufficient to obtain reproducible signal ratios over the 20–30 min duration of a typical experiment. There was little fura-2 leakage from HUVEC monolayers and minimal dye bleaching as judged by the stability of the signal intensity at 360 nm excitation (which is relatively [Ca++]i insensitive), during the course of an experiment. Although compartmentalization of fura-2 within HUVEC frequently occurred when these cells were loaded at 37°C (31), this problem was circumvented by use of the dispersing agent, Pluronic, and by loading and allowing HUVEC to hydrolyze fura-2 AM to fura-2 free acid at 18°C (27). Subsequent warming of HUVEC monolayers to 37°C for use in experiments did not result in compartmentalization of the dye. Only HUVEC which displayed a homogenous cytoplasmic distribution of fura-2 were used for measurements. Since incomplete fura-2 hydrolysis results in inaccurate [Ca++]i, measurements (15), completeness of dye hydrolysis was established by determining that the peak fluorescent signal emission occurred at 505–510 nm, by the stability of the 360 nm excitation signal, and by the ability of manganese to quench fura-2 fluorescence. Manganese and other divalent metal cations quench fura-2 free acid fluorescence but not that of the unhydrolyzed fura-2 ester. MnCl2 (10 mM) quenched virtually all of the fluorescent signal attributed to fura-2; the residual fluorescence was comparable to that of HUVEC which had not been loaded with fura-2.

Fura-2 Signal Ratios Are Calcium Sensitive and Yield Reproducible Cytosolic Free Calcium Measurements

To establish that the ratio of fura-2 fluorescence signal intensity at two excitation wavelengths reflected [Ca++]i, and to determine HUVEC [Ca++]i, fura-2 fluorescence was measured at maximum (saturating) and minimum [Ca++]i at the end of each experiment. Measurements in the presence of saturating [Ca++]i values among different HUVEC (≥20 cells/monolayer, 200–300 monolayers examined) within given monolayer ranged from 5–25% under resting conditions. These differences are likely to be due to differences in uptake and hydrolysis of fura-2 by individual cells. Therefore all results reported represent [Ca++]i measurements on a single HUVEC unless noted otherwise and only changes in [Ca++]i in excess of 25% from resting values are interpreted as significant.
Table I. Effect of Soluble Mediators on the Electrical Resistance of Human Umbilical Vein Endothelial Cell Monolayers

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>n</th>
<th>Change in Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>10^{-6} M</td>
<td>3</td>
<td>50 ± 10%*</td>
</tr>
<tr>
<td>Leukotriene C_{4}</td>
<td>10^{-6} M</td>
<td>4</td>
<td>44 ± 7%</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1 U/ml</td>
<td>4</td>
<td>65 ± 22%</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Transendothelial electrical resistance measurements were made in the presence of M199/FBS (baseline) and every 30 s for 30 min after the addition of the specified agonist to the apical and basal surfaces of HUVEC monolayers. Change in resistance is expressed as the maximal percentage decrease from baseline measurements during the 30 min incubation.

**Soluble Agonists Which Increase Human Umbilical Vein Endothelial Cell Monolayer Permeability Increase Human Umbilical Vein Endothelial Cell Cytosolic Free Calcium**

We have reported that histamine increases the permeability of HUVEC monolayers cultured on amnion to ions (17). Transendothelial electrical resistance of HUVEC monolayers on amnion also was measured before and during the incubation (30 min) of monolayers with LTC_{4} (10^{-4} M) and thrombin (1 U/ml). These substances all induced an increase in the permeability of HUVEC monolayers to ions as reflected by a decrease in electrical resistance (Table I). Their effect, both qualitatively and quantitatively, is in agreement with previous reports that histamine and thrombin increase the permeability of cultured EC monolayers to various molecules (10, 20, 30). HUVEC [Ca^{2+}], was measured under resting conditions and after the addition of each of the substances listed above to the apical surface of HUVEC monolayers. Histamine, LTC_{4}, and thrombin produced a transient 3-13-fold increase in HUVEC [Ca^{2+}], (Fig. 2). Others have reported that histamine increases HUVEC [Ca^{2+}], (19, 30). In the current studies, increases in HUVEC [Ca^{2+}], in response to these soluble agonists generally occurred within 15-30 s after the addition of these substances whereas the transendothelial electrical resistance of HUVEC monolayers did not decrease until 1.5 min after the addition of these substances. The correlation between the ability of substances to increase HUVEC [Ca^{2+}], and their ability to increase the permeability of HUVEC monolayers to ions suggests that increases in HUVEC [Ca^{2+}], may promote opening of intercellular junctions between HUVEC, thereby increasing the permeability of HUVEC monolayers.

**Polymorphonuclear Leukocyte Migration across a Human Umbilical Vein Endothelial Cell Monolayer Induces a Five to Eightfold Transient Increase in Human Umbilical Vein Endothelial Cell Cytosolic Free Calcium**

[Ca^{2+}], in single HUVEC was measured as described above under resting conditions and for 30 min after the initiation of PMN migration across the HUVEC monolayer. Since PMN attach randomly to EC in a monolayer and <20% of PMN migrate across the monolayer in response to a chemoattractant (9, 13, 17), 10 PMN were added for each HUVEC in the monolayer (2 x 10^{4} PMN/2 x 10^{5} HUVEC) in most studies to increase the likelihood that each HUVEC would establish contact with a migrating PMN. PMN resuspended in HBS/HIFBS or HBS/BSA were first added to the luminal side of a HUVEC monolayer in the absence of a chemoattractant. Under this condition, PMN did not induce a significant change in resting HUVEC [Ca^{2+}], (Fig. 3, Table II). When fMLP (10^{-7} M) was then added to the compartment beneath the HUVEC monolayer, PMN migration was stimulated and induced a transient five to eightfold increase in HUVEC [Ca^{2+}], (n = 10) (Fig. 3, Table II). This increase in HUVEC [Ca^{2+}], temporally corresponded to the time course of transendothelial PMN migration (17). Under these conditions, 2.3 ± 0.3 PMN for each HUVEC in the monolayer migrated from the luminal to the abluminal aspect of the HUVEC monolayer as assessed by light microscopy (Table III). Migrating PMN, at a PMN:EC ratio of 5:1, induced a transient two to threefold increase in HUVEC [Ca^{2+}], (data not shown). Neither the physical manipulation of replacing the medium above HUVEC monolayers nor the addition of fMLP alone below HUVEC monolayers induced a significant change in resting HUVEC [Ca^{2+}], (Table II), indicating that the rise in [Ca^{2+}], was induced by the fMLP-stimulated PMN and not by an effect of fMLP on EC.
Figure 3. Representative curve of HUVEC [Ca++] during fMLP stimulated PMN migration across a HUVEC monolayer (n = 10). HUVEC monolayers on amnion were loaded with fura-2 and [Ca++] measurements were made in single cells maintained in intact monolayers. HUVEC [Ca++] was measured in the presence of HBS/HIFBS or HBS/BSA, after the addition of PMN alone to the apical surface of the HUVEC monolayer (PMN:HUVEC ratio = 10:1), and after the perfusion of fMLP (10^-7 M) below the HUVEC monolayer. PMN migration across the HUVEC monolayer occurred during the interval indicated on the horizontal axis. 2.3 ± 0.3 PMN for each HUVEC migrated under these conditions.

Polymorphonuclear Leukocyte Adhesion to Human Umbilical Vein Endothelial Cells Increases Human Umbilical Vein Endothelial Cell Cytosolic Free Calcium and Increases the Permeability of Human Umbilical Vein Endothelial Cell Monolayers to Ions

We tested whether PMN adhesion to HUVEC was sufficient to induce a change in HUVEC [Ca++], or whether directed PMN migration through an endothelium was required to induce such changes. HUVEC [Ca++] was measured after the addition of both PMN and fMLP to the apical surface of HUVEC monolayers.

Table II. Human Umbilical Vein Endothelial Cell [Ca++], During Polymorphonuclear Leukocyte Migration

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>HUVEC [Ca++] [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>10</td>
<td>108 ± 17*</td>
</tr>
<tr>
<td>2. Addition of PMN above HUVEC (no chemoattractant)</td>
<td>6</td>
<td>129 ± 18</td>
</tr>
<tr>
<td>3. Maximal [Ca++], after addition of fMLP below HUVEC in the presence of PMN above HUVEC</td>
<td>10</td>
<td>769 ± 200</td>
</tr>
<tr>
<td>4. Conditions as in 3 above, but 15 min after the onset of PMN migration</td>
<td>10</td>
<td>160 ± 49</td>
</tr>
<tr>
<td>5. Addition of fMLP to the basal surface of HUVEC</td>
<td>4</td>
<td>109 ± 24</td>
</tr>
<tr>
<td>6. Addition of fMLP to the apical surface of HUVEC</td>
<td>2</td>
<td>116 ± 14</td>
</tr>
</tbody>
</table>

* Mean ± SD.
HUVEC monolayers on amnion were loaded with fura-2 and [Ca++], measurements were made in single HUVEC maintained in intact monolayers under the specified conditions.

Table III. Polymorphonuclear Leukocyte Migration across Human Umbilical Vein Endothelial Cell Monolayers

<table>
<thead>
<tr>
<th>Agonist/condition</th>
<th>Target cell</th>
<th>n</th>
<th>PMN/HUVEC associated with HUVEC monolayer</th>
</tr>
</thead>
<tbody>
<tr>
<td>fMLP (10^-7 M)</td>
<td>PMN</td>
<td>10</td>
<td>2.3 ± 0.3 migrated beneath HUVEC</td>
</tr>
<tr>
<td>fMLP (10^-7 M)</td>
<td>PMN</td>
<td>2</td>
<td>&lt; 0.1 migrated beneath HUVEC</td>
</tr>
<tr>
<td>Interleukin 1 (5 U/ml)</td>
<td>HUVEC</td>
<td>2</td>
<td>2.4 ± 0.6 migrated beneath HUVEC</td>
</tr>
</tbody>
</table>

* Mean ± SD.
† fMLP and PMN were added to the apical surface of HUVEC monolayers.
‡ The apical surface of HUVEC monolayers was incubated with IL-1 for 4 h at 37°C, then washed before adding PMN. No IL-1 or fMLP was present during migration assay.
§ A ratio of 10 PMN for each HUVEC was added to the apical surface of HUVEC monolayers and fMLP was added below HUVEC monolayers as a chemoattractant except where noted. PMN and HUVEC were incubated together for 15 min at 37°C, fixed, and stained as indicated in Materials and Methods. PMN adhesion and/or migration was quantified by light microscopy.

HUVEC monolayers. Under these conditions, 1.5 ± 0.3 PMN adhered to the apical surface of each HUVEC but <0.1 PMN/HUVEC migrated beneath the monolayer (Table III). The interaction of fMLP-stimulated PMN with HUVEC induced a transient three to fivefold increase in HUVEC [Ca++], (n = 4) (Fig. 4 a). These findings make it unlikely that the changes in HUVEC [Ca++], which occur in response to PMN-HUVEC interactions are a secondary effect of transendothelial PMN migration or due to physical changes in the HUVEC during this process. The addition of fMLP alone to the apical surface of HUVEC monolayers had no effect on HUVEC [Ca++]. (Table II).

Because PMN adhesion to HUVEC increased HUVEC [Ca++], we also determined whether PMN-HUVEC adhesion increased the permeability of HUVEC monolayers. Electrical resistance measurements were made as described in Materials and Methods under the experimental conditions described in the preceding paragraph. Average transendothelial electrical resistance of HUVEC monolayers was 12 ± 13 Ω·cm². The interaction of fMLP-stimulated PMN with HUVEC induced a 45 ± 10% decrease in HUVEC resistance (n = 5) (Fig. 4 b). These findings further correlated increases in HUVEC [Ca++] with increases in HUVEC monolayer permeability and suggested a role for HUVEC [Ca++], in regulating the integrity of junctions between EC. FMLP stimulated PMN induced increases in HUVEC [Ca++], and HUVEC monolayer permeability in the presence of both HBS/HIFBS as well as HBS/BSA. The lack of effect of HIFBS on PMN induced increases in HUVEC [Ca++], and monolayer permeability suggests that oxidants and/or proteases secreted by PMN are not the effectors of these changes.

While there is an excellent correlation between agents that increase EC [Ca++], (Figs. 2 and 4 a) and those that cause a decrease in electrical resistance of EC monolayers (Table I and Fig. 4 b), we have not observed a consistent relationship between the magnitude of the increase in [Ca++], and
IL-1 or tumor necrosis factor promote transendothelial migration of PMN (8, 29). We investigated whether EC initiated PMN migration also induces an increase in HUVEC [Ca++]. HUVEC monolayers were treated with IL-1 as described in Materials and Methods and then loaded with fura-2. After 4 h of exposure to IL-1, HUVEC [Ca++] was 94 ± 22 nM (n = 5), not significantly different from untreated cells. PMN then were added to the apical surface of HUVEC monolayers in the absence of added chemoattractant. HUVEC [Ca++] increased beginning 2 min after adding PMN, increased sixfold within the next 5 min, and then returned to baseline within the subsequent 3–5 min (Fig. 5). This increase in HUVEC [Ca++], also temporally corresponded with the time course of transendothelial PMN migration. The time course and magnitude of PMN migration across IL-1 treated endothelium is reported to be similar to that seen when fMLP stimulated PMN migrate across monolayers of untreated HUVEC (8). Under the conditions employed here, 2.4 ± 0.6 PMN for each IL-1 treated HUVEC migrated across the monolayer (Table III).

PMN (PMN:EC ratio = 10:1) migration across IL-1 treated HUVEC monolayers induced no change in transendothelial electrical resistance (n = 3) but larger numbers of PMN (PMN:EC ratio = 25:1) induced a 40 ± 2% decrease in electrical resistance (n = 3) (see Discussion). Under these conditions, 1.9 ± 0.6 and 4.6 ± 0.8 PMN, respectively, for each IL-1 treated HUVEC migrated across the monolayer. PMN induced an increase in HUVEC [Ca++], and a decrease in electrical resistance in monolayers of IL-1 treated HUVEC in the presence of both HBS/HIFBS as well as HBS/BSA.

MAPTAM Clamps Human Umbilical Vein Endothelial Cell Cytosolic Free Calcium at Resting Levels in Response to Histamine and Stimulated Polymorphonuclear Leukocytes

To determine whether the observed transient increases in
Figure 6. Effect of MAPTAM on histamine induced increases in HUVEC [Ca++] (n = 3). HUVEC monolayers were incubated with HBS/FBS (2%) with or without MAPTAM (200 μM) for 30 min at 37°C, washed, then loaded with fura-2. Representative curves are shown of [Ca++] in the presence of HBS/BSA and following the addition (arrow) of histamine (10^-5 M) in MAPTAM treated (●) and control (○) HUVEC.

HUVEC [Ca++] play a role in transendothelial PMN migration, we examined the effect of clamping HUVEC [Ca++] at resting levels on the ability of PMN to migrate across monolayers of HUVEC in response to fMLP or IL-1 treated HUVEC. HUVEC monolayers were incubated in calcium replete buffer containing MAPTAM, a cell permeant calcium buffer, then incubated with fura-2 as described under Materials and Methods. HUVEC [Ca++] was 99 ± 26 nM after a 30-min incubation with MAPTAM and 83 ± 24 nM in control cells (n = 5). Histamine (10^-5 M) induced a transient four to fivefold increase in HUVEC [Ca++], in control cells but no significant change in MAPTAM treated cells (n = 3) (Fig. 6). Pretreatment of HUVEC monolayers with MAPTAM similarly blocked increases in HUVEC [Ca++], induced by fMLP-treated PMN. HUVEC [Ca++] was 113 ± 23 (n = 4) after a 30-min incubation with MAPTAM and did not increase in response to addition of PMN (at PMN:EC ratios of 5:1 and 10:1) to the upper chamber and fMLP to the lower chamber. To verify that the fura-2 was hydrolyzed and that fura-2 provided an accurate measure of [Ca++], in MAPTAM treated cells, fura-2 fluorescence was measured in the presence of saturating and minimum [Ca++], and MnCl2 as described above under Results. There was no significant difference in the fura-2 fluorescence signals under these conditions between MAPTAM treated and control cells.

Clamping Human Umbilical Vein Endothelial Cell Cytosolic Free Calcium at Resting Levels Inhibits Transendothelial Polymorphonuclear Leukocyte Migration

EC monolayers were loaded with MAPTAM, as described in Materials and Methods, washed several times to remove MAPTAM, and did not incubate in response to addition of intracellular MAPTAM as described above for [Ca++], experiments. PMN, at a ratio of 5 PMN/EC were added above the EC monolayers and allowed to migrate for 30 min in response to an fMLP chemotactic gradient or IL-1 treated HUVEC. The number of PMN associated with each monolayer was quantified as described in Materials and Methods. In response to an fMLP gradient, 58 ± 4 PMN/400x field were associated with monolayers maintained under control conditions, 26 ± 3 PMN were associated with MAPTAM treated monolayers (n = 3). In response to IL-1 treated HUVEC monolayers, 87 ± 6 and 55 ± 3 PMN/400x field were associated with control monolayers and MAPTAM treated monolayers respectively (n = 3). Cross-sections of samples were examined to determine whether the PMN associated with the monolayer under these conditions had migrated across the HUVEC monolayer or were adherent to the apical surface of HUVEC. The positions of >700 PMN were determined under each condition. In response to fMLP, >80% of the PMN associated with monolayers under control conditions, but <21% of the PMN associated with MAPTAM treated monolayers had migrated across the HUVEC monolayers and into the collagenous matrix of the underlying amnion (Fig. 7). In response to IL-1 treated HUVEC, >70% of the PMN associated with the monolayer under control conditions, but <15% of the PMN associated with MAPTAM treated monolayers had migrated across the HUVEC monolayers. Thus, MAPTAM treatment of HUVEC monolayers inhibited transendothelial cell PMN migration by >89% in response to fMLP and by >87% in response to IL-1 treated HUVEC (Fig. 8 a).
Published March 15, 1993

The Journal of Cell Biology, Volume 120, 1993 1378

PMN adhesion and/or migration to MAPTAM treated HUVEC monolayers. 15 min after clamping HUVEC monolayers were incubated with or without MAPTAM as described under Materials and Methods. PMN and HUVEC were incubated together at 37°C, fixed, and processed as described in Materials and Methods. PMN adhesion and/or migration was quantified by examining cultures en face and in cross section under light microscopy. (a) The number of PMN migrating across control (open bars) or MAPTAM treated (hatched bars) HUVEC monolayers was calculated from the percentage of PMN located below HUVEC monolayers on cross section and the total number of PMN associated with monolayers en face. (b) The number of PMN adhering to control (open bars) or MAPTAM treated (hatched bars) HUVEC monolayers is shown.

Since PMN adhesion to EC is a prerequisite for transendothelial migration, we determined whether clamping HUVEC [Ca++] at resting levels affected the number of PMN adhering to MAPTAM treated HUVEC monolayers. 15 min after the addition of both PMN and fMLP to the apical surface of these monolayers, there was no significant difference in PMN adherence to control vs MAPTAM treated HUVEC. 59 ± 14 PMN/400× field adhered to MAPTAM treated HUVEC monolayers and 61 ± 15 PMN/400× field adhered to control monolayers (n = 2) (Fig. 8 b). PMN-EC adhesion was assessed at 15 min because previous studies indicated that maximum adhesion occurs at this time (9, 33). PMN migration experiments were conducted for 30 min to facilitate the measurement of the position of PMN above or below HUVEC monolayers. By 30 min, many PMN that had adhered to EC but were unable to migrate across EC monolayers had detached, thus, accounting for the smaller number of PMN associated with MAPTAM treated HUVEC monkeyers in migration experiments.

To determine whether the inhibition of PMN migration across MAPTAM-treated HUVEC monolayers could have been due to an effect of any residual MAPTAM on PMN, we examined PMN migration into MAPTAM-treated amnion membranes without HUVEC monolayers. Amniotic membranes without HUVEC monolayers were preincubated in HBS with or without MAPTAM and washed to remove the MAPTAM as described under Materials and Methods. PMN were then added above the amniotic membranes and PMN migration was stimulated by placing fMLP below the amnion in the standard fashion. There was no difference in PMN migration into MAPTAM treated and control amnion in response to an fMLP gradient (data not shown).

Clamping Human Umbilical Vein Endothelial Cell Cytosolic Free Calcium at Resting Levels Reduces Polymorphonuclear Leukocyte Induced Increases in Human Umbilical Vein Endothelial Cell Monolayer Permeability

To determine whether PMN induced increases in HUVEC [Ca++] affect transendothelial PMN migration by opening junctions between HUVEC, we examined the effect of clamping HUVEC [Ca++], at resting levels on PMN induced decreases in the electrical resistance of HUVEC monolayers. Because monolayers preincubated with Pluronic and DMSO or Pluronic, DMSO, and MAPTAM exhibited a decreased electrical resistance, we preincubated the HUVEC monolayers to be used in these experiments in medium containing IBMX and forskolin with or without MAPTAM as described in Methods. Under these conditions, both control and MAPTAM treated monolayers had baseline resistances of 13 ± 2 Ω·cm². Monolayers were washed to remove IBMX, forskolin, and MAPTAM, and incubated in fresh medium. PMN at a PMN:EC ratio of 10:1 and fMLP were added to the apical surface of these monolayers and their electrical resistance monitored. fMLP stimulated PMN induced a 22 ± 6% decrease in the resistance of control monolayers (n = 3) but only a 8 ± 4% decrease in the resistance of MAPTAM treated monolayers (n = 3). Thus clamping HUVEC [Ca++], at resting levels significantly reduced PMN induced increases in HUVEC monolayer permeability to ions. These findings indicate that by promoting increases in HUVEC [Ca++], PMN are able to influence the integrity of junctions between HUVEC.

Discussion

These studies identify three previously unrecognized aspects of transendothelial migration of PMN. First, they demonstrate that PMN stimulated by chemoattractants or by cytokine activated EC signal a rise in EC [Ca++]. Second, they show that this rise in [Ca++] is not required for adhesion of chemoattractant-stimulated PMN to EC, but is required for migration of these PMN across an EC monolayer. Third, they show that by inducing an increase in EC [Ca++], PMN are able to affect the integrity of junctions between EC. Viewed together with data from other laboratories (14, 30, 34) on the effects of increased [Ca++], on EC shape and on the integrity of interendothelial cell junctions, our findings suggest that EC are active as opposed to passive participants in the transendothelial migration of PMN.

Stimulated Polymorphonuclear Leukocytes Induce an Increase in Endothelial Cell [Ca++].

We selected EC [Ca++], as an indicator of trans-cellular signaling between PMN and EC because of the general importance of [Ca++], as a second messenger and because of its involvement in cytoskeletal functions. A rise in EC [Ca++], is associated with myosin light chain phosphorylation, cytoskeletal reorganization, and opening of intercellular junctions (30, 34). Our studies show that migration of fMLP-stimulated PMN across monolayers of untreated EC, and of
unstimulated PMN across monolayers of IL-1 treated EC, induces an increase in EC \([\text{Ca}^{++}]\), that coincides temporally with the migration of PMN from the luminal to the abluminal surface of the EC monolayer.

That PMN induce the same EC intracellular signaling event in response to fMLP or to cytokine-treated EC is not surprising because in both instances PMN are responding to a soluble chemoattractant. During fMLP-stimulated PMN migration, the fMLP itself is the soluble chemoattractant. During PMN migration across cytokine-stimulated (e.g., IL-1 or TNF) endothelium, chemoattractants such as IL-8 (32) and platelet activating factor (21) that are produced by EC promote transendothelial PMN migration in the absence of added chemoattractant (18).

**Role of Stimulated Polymorphonuclear Leukocytes in Mediating an Increase in Permeability of Endothelial Cell Monolayers**

Our finding that the adhesion of stimulated PMN to the apical surface of EC monolayers decreases the electrical resistance of EC monolayers appears to contradict a previous report from our laboratory. We reported that PMN migration across HUVEC monolayers (PMN:EC ratio = 5:1) in response to fMLP or leukotriene B4 occurs without measurable changes in the permeability of these monolayers to ions (17). This lack of change in permeability was explained on a structural basis. Ions traverse an EC monolayer through the intercellular spaces, also known as the paracellular pathway (6). At a PMN:EC ratio of 5:1, the increase in the dimensions of these spaces during PMN migration is compensated by the presence of PMN in the intercellular spaces and the closeness of apposition between PMN and EC (<150Å). In contrast, at higher PMN:EC ratios (25–50:1) (17), or when PMN adhered to the EC monolayer but did not migrate across it (Fig. 4 b), we observed a 12–50% decrease in the transendothelial electrical resistance of these monolayers. This is consistent with our hypothesis that a rise in EC \([\text{Ca}^{++}]\), promotes opening of junctions between EC and that at lower PMN:EC ratios the presence of PMN in these spaces "plugs" the gap between the EC. At higher PMN:EC ratios, however, a greater increase in the size of the paracellular pathway in EC monolayers is created by the presence of larger numbers of PMN residing in the intercellular spaces during transendothelial passage. This increase in size of the paracellular pathway is larger than can be compensated by the closeness of apposition between PMN and EC (reference 17, Fig. 10). The result is an increase in the permeability of the monolayer to ions. When PMN are unable to migrate across the EC monolayer because there is no chemotactic gradient directing them from the monolayer's luminal to abluminal side (Fig. 4 b), PMN are not attracted to the gaps that form between EC as a result of signals sent to the EC by the chemoattractant-stimulated PMN, and the permeability of the monolayer to ions is increased.

**Role of Endothelial Cell \([\text{Ca}^{++}]\) in Transendothelial Migration of Stimulated Polymorphonuclear Leukocytes**

PMN require physiological concentrations of \(\text{Ca}^{++}\) in the medium in order to bind to their cognate ligands on EC (16). Similarly, physiological concentrations of \(\text{Ca}^{++}\) in the medium are needed to maintain the integrity of junctions between EC (7, 17). For these reasons, it was not possible to perform experiments in \(\text{Ca}^{++}\)-free solutions. Therefore we used a cell-permeant \(\text{Ca}^{++}\) chelator (MAPTAM) to clamp \([\text{Ca}^{++}]\), in EC at resting levels (<100 nM), while maintaining these cells in \(\text{Ca}^{++}\) replete (1 mM) medium. Experiments showed that such MAPTAM-treated EC exhibited no increase in \([\text{Ca}^{++}]\), when stimulated with histamine (Fig. 6) or activated PMN (see Results).

Chemoattractant-stimulated PMN adhered equally to control EC monolayers and to EC monolayers whose \([\text{Ca}^{++}]\), was clamped at resting levels by MAPTAM (Fig. 8 b). However, MAPTAM treatment of these monolayers inhibited transendothelial movement of the PMN (Figs. 7 and 8 a). Control experiments demonstrated that chemoattractant-stimulated PMN migrated normally into the collagenous matrix of amniotic membranes that had been preincubated with MAPTAM and then washed to remove this \(\text{Ca}^{++}\) chelator before addition of the PMN. More importantly, Kuijpers et al. (22) showed that buffering of \([\text{Ca}^{++}]\), in PMN had no inhibitory effect on the capacity of PMN to migrate across EC monolayers in response to fMLP or to cytokine-treated endothelium. Therefore, it is unlikely that the inhibition of transendothelial PMN migration by MAPTAM-treated EC resulted from the effects of residual MAPTAM on PMN. Taken together, these results lead us to conclude that an increase in EC \([\text{Ca}^{++}]\), is necessary for PMN to migrate across endothelia.

**A Unifying Hypothesis**

How might changes in EC \([\text{Ca}^{++}]\), regulate transendothelial movement of PMN? We propose the following: EC retraction, similar to the contraction of smooth muscle and nonmuscle cells such as platelets, is associated with the phosphorylation of myosin light chains by myosin light chain kinase. This event is \(\text{Ca}^{++}\) and calmodulin dependent (I). Activation of EC myosin causes these cells to retract (34). EC \([\text{Ca}^{++}]\), transients also may signal uncoupling of adhesive or occluding junctions between EC. Either or both of these events would facilitate the opening of junctions between EC and the movement of PMN from the intravascular to the extravascular compartment.

The requirement for intercellular signaling for PMN to traverse endothelia is unlikely to be restricted to these two cell types. Similar events probably occur when leukocytes traverse epithelia, when cancer cells invade, and when one cell migrates through a field of cells during development of an organ or tissue. In this sense, we believe the mechanisms we have described for PMN and endothelia may provide an example of a general paradigm for cooperation between cells during cell migration.

The authors would like to thank Mr. Llewellyn Ward and Ms. Arline Albal for their generosity and assistance in preparing paraffin embedded sections.

Supported by National Institutes of Health Clinical Investigator Award HL02202 to A. J. Huang, grant HL32210 to S. C. Silverstein, the Cystic Fibrosis Research Development Program at Columbia University, New York, and a generous gift of Mr. Samuel W. Rover.

Received for publication 28 August 1992 and in revised form 16 December 1992.
References


