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

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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 ($[Ca^{++}]_i$) (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 C_4 (LTC_4) and thrombin, which increased the permeability of EC monolayers to ions, as measured by the electrical resistance of the monolayers, transiently increased EC $[Ca^{++}]_i$. To determine whether chemotaxing PMN cause similar changes in EC $[Ca^{++}]_i$, human umbilical vein endothelial cells (HUVEC) maintained as monolayers were loaded with fura-2. $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ which temporally corresponded with the time course of PMN-EC interactions. When EC $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ did not inhibit PMN-EC adhesion. These studies provide evidence that EC respond to stimulated PMN by increasing their $[Ca^{++}]_i$ and that this increase in $[Ca^{++}]_i$ causes an increase in EC monolayer permeability. Such $[Ca^{++}]_i$ increases are required for PMN transit across an EC barrier. We suggest EC $[Ca^{++}]_i$ 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)[1] 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 ($[Ca^{++}]_i$), 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

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1. *Abbreviations used in this paper*: $[Ca^{++}]_i$, 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; LTC_4, leukotriene C_4; M199/FBS, M199 medium containing 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml amphotericin B; PMN, polymorphonuclear leukocytes.

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(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 $[Ca^{++}]_i$ transients.

To examine whether PMN interactions with EC induce a change in EC $[Ca^{++}]_i$, we developed a method for measuring $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ and that inhibition of this rise in $[Ca^{++}]_i$ 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 C_4 (LTC_4) 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 replated on the stromal surface of human amniotic 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 μg/ml streptomycin, and 2 μg/ml amphotericin B (M199/FBS). HUVEC cultures on amnion reached confluence within 2 d and were used from 7–12 d after plating. Previous studies demonstrated that such cultures exhibit maximum transendothelial electrical resistance (6-12 $\Omega \cdot cm^2$) 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% NaCl bridges were used. 10 microamperes of DC current were passed though one pair of electrodes in both the luminal to abluminal and abluminal to luminal directions for all measurements. The voltage drop across the HUVEC on amnion was measured with the second pair of electrodes and recorded. Resistance of the HUVEC-amnion culture was calculated from these two known values using Ohm's law (V = IR). Background resistance of the amnion and medium or buffer bathing the HUVEC but without the HUVEC monolayers was measured for each sample and subtracted from the resistance measurements of HUVEC-amnion cultures to obtain the electrical resistance due to HUVEC monolayers alone. In all instances, the resistance observed was the same regardless of the direction of current flow.

In experiments which examined the effect of MAPTAM on PMN-induced increases in HUVEC monolayer permeability, preincubation of HUVEC monolayers with Pluronic and DMSO (controls) decreased the transendothelial electrical resistance of HUVEC monolayers by 15–20%. Both control and MAPTAM treated HUVEC monolayers exhibited similar decrease in resistance and this reduction in resistance had no effect on transendothelial migration of PMN. However, this decrease in resistance made it difficult to determine if there was a significant difference in PMN induced decreases in resistance between control and MAPTAM treated monolayers. For this reason, HUVEC monolayers used in these experiments were incubated with isobutyl methylxanthine (IBMX) (1 mM) and forskolin (25 μM) (23) for 90 min at 37°C before adding PMN and fMLP. IBMX and forskolin were not present in the medium during electrical resistance measurements following the addition of PMN and fMLP. Incubation of HUVEC monolayers with IBMX and forskolin raised transendothelial resistance from an average of 7 ± 2 $\Omega \cdot cm^2$ to 13 ± 2 $\Omega \cdot cm^2$. HUVEC monolayer resistance remained constant in the absence of IBMX and forskolin for at least the 15 min required for a typical experiment.

Polymorphonuclear Leukocyte Isolation and Migration

Human whole blood was obtained by venipuncture after informed consent and peripheral blood neutrophils were isolated on a discontinuous Ficoll-Hypaque (Sigma Chemical Co.) gradient (5). Purified PMN were resuspended at 4×10^6 cells/ml in Hepes buffered saline (20 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, pH 7.4) (HBS) containing 20% heat inactivated FBS (HBS/HIFBS) or BSA (5 mg/ml) (Sigma Chemical Co.) and held on ice (<3 h) until use.

PMN were added above HUVEC monolayers and transendothelial PMN migration was stimulated by placing formyl-methionyl-leucyl-phenylalanine (fMLP) (Peninsula Laboratories, Belmont, CA) (10^{-7} M) in HBS/HIFBS on the opposite side of the monolayer or by incubating the apical surface of HUVEC monolayers with M199/FBS containing recombinant IL-1 β (Cistron Technology, Pinebrook, NJ) (5 u/ml) 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 $[Ca^{++}]_i$ 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 removed from Teflon rings and stained with tetrachrome blood stain (Harleco, Gibbstown, NJ). Stained cultures were mounted on slides and the number of PMN that had migrated was visually assessed by light microscopy. To prepare cross-sections, cultures were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3, dehydrated, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin.

Cytosolic Free Calcium Measurements

Fura-2 AM (5 μM) (Molecular Probes, Inc., Eugene, OR), Pluronic (0.02%) (Molecular Probes, Inc.), and FBS (2%) in HBS was prepared by sequentially adding each of these reagents in the specific order, mixing thoroughly after each addition. The apical and basal surfaces of a HUVEC monolayer cultured on amnion were incubated with 0.5 and 1.0 ml respectively 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 HBS and incubated in HBS 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 $[Ca^{++}]_i$ was clamped, HUVEC monolayers were first incubated with HBS containing FBS (2%), Pluronic (0.02%), and the cell permeant calcium buffer, MAPTAM, (200 μM) (Calbiochem Corp., San Diego, CA) for 30 min at 37°C, washed, and then loaded with fura-2 as described above.

HUVEC-amnion cultures were mounted in a lucite chamber and bathed in HBS containing BSA (5 mg/ml) (HBS/BSA) unless otherwise indicated. This chamber was maintained at 37°C with a perfusion water jacket and the entire apparatus (migration chamber and water jacket) was mounted on the stage of an upright Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Excitation wavelength was determined by placing narrow band width filters (either 380 ± 5 nm or 350 ± 5 nm) between the light source and the HUVEC. Emission wavelength was determined by a 510 ± 15 nm filter placed between the HUVEC and the photomultiplier tube (Fig. 1). A pinhole stop positioned between the HUVEC and the photomultiplier tube determined the size of the field from which fura-2 fluorescence was measured. In all experiments reported here, unless otherwise noted, measurements were obtained on a single HUVEC maintained within an intact monolayer. A photomultiplier tube (Carl Zeiss, Oberkochen, West Germany) attached to the microscope amplified and measured the fluorescence emitted by fura-2 loaded HUVEC and transmitted these signals to a MSP 20 microscope system processor (Carl Zeiss, Oberkochen). The processor converted the signals into digital values, calculated the ratio of fluorescence intensity at two different excitation wavelengths, and transmitted these values to a computer.

Standardization of Calcium Measurements

$[Ca^{++}]_i$ was calculated from ratios of fura-2 fluorescence at two excitation wavelengths as described by Grynkiewicz (11). The K_D of fura-2 for Ca^{++} at 37°C based on measurements made at 340 and 380 nm with this photometry system was 213 nM which is in excellent agreement with the value of 224 nM reported by Grynkiewicz (11). Due to the relative lack of light trans-

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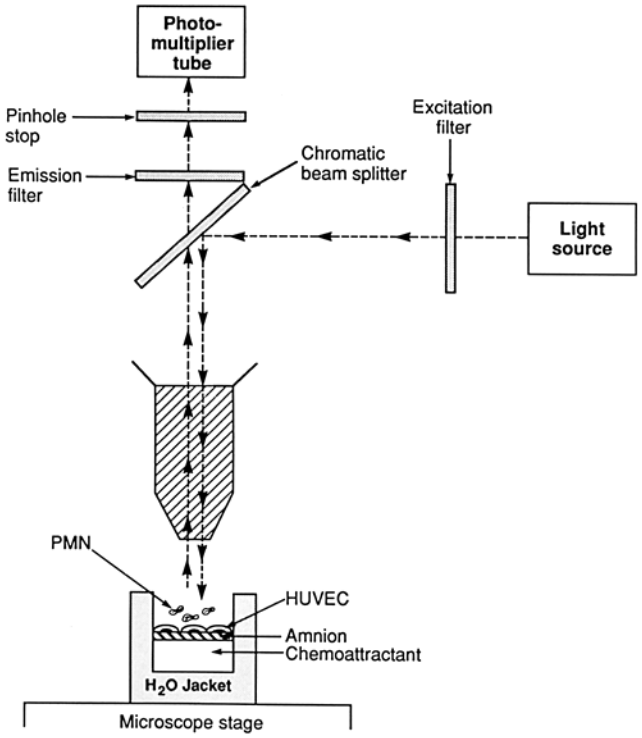

Figure 1. Schematic drawing of apparatus for making $[Ca^{++}]_i$ measurements in single HUVEC maintained in intact monolayers. HUVEC on amnion were loaded with fura-2 and mounted in a water jacketed chamber on the stage of an upright fluorescence microscope which was equipped with a photomultiplier 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 and 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 K_D 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. $MnCl_2$ (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 $[Ca^{++}]_i$ were made by incubating both luminal and abluminal surfaces of the HUVEC monolayer in HBS containing $CaCl_2$ (3 mM) and the calcium ionophore, ionomycin (1 μM) (24). Measurements in the presence of minimum $[Ca^{++}]_i$ were made by washing the HUVEC monolayers three times with HBS without divalent cations and incubating them in divalent cation-free HBS containing EGTA (2 mM) and ionomycin (1 μM). In this manner, each experiment served as its own control and demonstrated that the fluorescence signal responded appropriately to changes in $[Ca^{++}]_i$. The autofluorescence of HUVEC on amnion was measured after the addition of $MnCl_2$ (10 mM) which quenched fluorescence due to fura-2. This autofluorescence represented 5–10% of the total fluorescence signal of fura-2-loaded HUVEC under resting conditions and was subtracted from all measurements to yield the final ratio of fura-2 fluorescence intensity from which $[Ca^{++}]_i$ values were calculated.

Using this method, average resting HUVEC $[Ca^{++}]_i$ was 112 ± 20 nM (n = 30) and varied ⩽8% within a single cell over a 30-min period. This value is in excellent agreement with that reported for EC of bovine and human origin (3, 25, 30), and for many other mammalian cells. To determine the effect of cell selection within the HUVEC monolayer on $[Ca^{++}]_i$ measurements, the variation in HUVEC $[Ca^{++}]_i$ among different cells in a monolayer was examined. The variation in fluorescence intensity ratios and therefore $[Ca^{++}]_i$ 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.

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Table I. Effect of Soluble Mediators on the Electrical Resistance of Human Umbilical Vein Endothelial Cell Monolayers

Agonist	Concentration	n	Change in Resistance (%)
Histamine	10^{-6} M	3	↓ 50 ± 10%*
Leukotriene C_4	10^{-8} M	4	↓ 44 ± 7%
Thrombin	1 U/ml	4	↓ 65 ± 22%

* 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^{-8} 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ (Fig. 2). Others have reported that histamine increases HUVEC $[Ca^{++}]_i$ (19, 30). In the current studies, increases in HUVEC $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ and their ability to increase the permeability of HUVEC monolayers to ions suggests that increases in HUVEC $[Ca^{++}]_i$ 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

$[Ca^{++}]_i$ 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 (2×10^6 PMN/2×10^5 HUVEC) in most studies to increase the likelihood that each HUVEC would

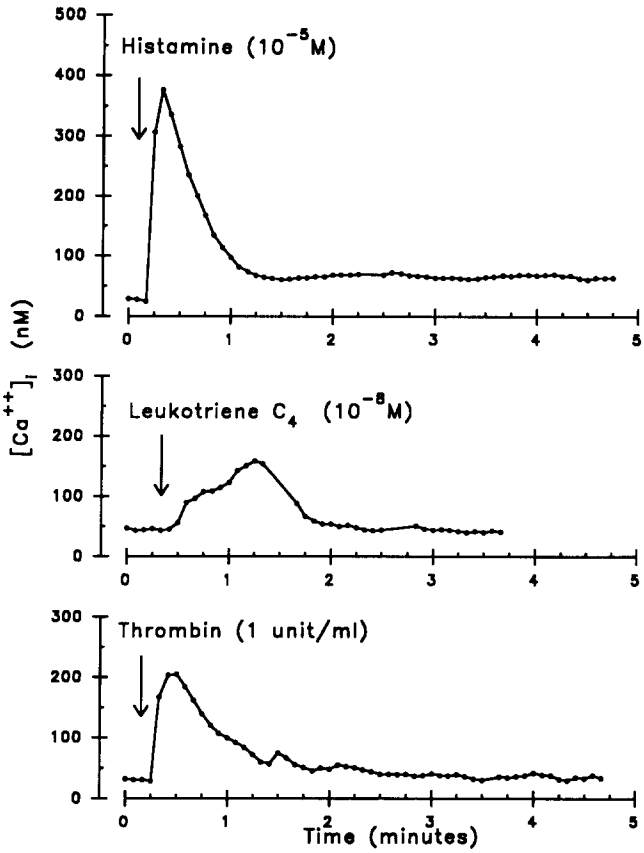

Figure 2. HUVEC $[Ca^{++}]_i$ in response to soluble agonists. Representative curves are shown of HUVEC $(Ca^{++})_i$ measured in single cells in fura-2 loaded HUVEC monolayers. $[Ca^{++}]_i$ was measured in the presence of HBS/BSA and every 5 s for 5 min after the addition (*arrows*) of (*a*) histamine (10^{-5} M), (*b*) LTC_4 (10^{-8} M), and (*c*) thrombin (1 unit/ml) to the apical surface of each monolayer. Each curve shown is representative of a minimum of three separate experiments.

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 $[Ca^{++}]_i$ (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 $[Ca^{++}]_i$ ($n = 10$) (Fig. 3, Table II). This increase in HUVEC $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ (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 $[Ca^{++}]_i$ (Table II), indicating that the rise in $[Ca^{++}]_i$ was induced by the fMLP-stimulated PMN and not by an effect of fMLP on EC.

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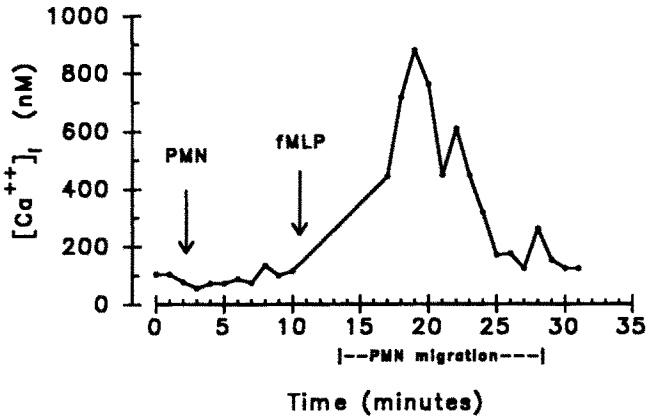

Figure 3. Representative curve of HUVEC $[Ca^{++}]_i$ during fMLP stimulated PMN migration across a HUVEC monolayer (n = 10). HUVEC monolayers on amnion were loaded with fura-2 and $[Ca^{++}]_i$ measurements were made in single cells maintained in intact monolayers. HUVEC $[Ca^{++}]_i$ was measured in 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 $[Ca^{++}]_i$ or whether directed PMN migration through an endothelium was required to induce such changes. HUVEC $[Ca^{++}]_i$ was measured after the addition of both PMN and fMLP to the apical surface of 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 $[Ca^{++}]_i$ (n = 4) (Fig. 4 *a*). These findings make it unlikely that the changes in HUVEC $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ (Table II).

Table II. Human Umbilical Vein Endothelial Cell $[Ca^{++}]_i$ During Polymorphonuclear Leukocyte Migration

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

* Mean ± SD.
HUVEC monolayers on amnion were loaded with fura-2 and $[Ca^{++}]_i$ 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

Agonist/condition	Target cell	n	PMN/HUVEC associated with HUVEC monolayer
fMLP (10^{-7} M)	PMN	10	2.3 ± 0.3* migrated beneath HUVEC
fMLP (10^{-7} M)‡	PMN	2	< 0.1 migrated beneath HUVEC 1.5 ± 0.3 adherent to apical surface of HUVEC
Interleukin 1 (5 U/ml)§	HUVEC	2	2.4 ± 0.6 migrated beneath HUVEC

* 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.

Because PMN adhesion to HUVEC increased HUVEC $[Ca^{++}]_i$, 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 $\Omega \cdot cm^2$. 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 $[Ca^{++}]_i$ with increases in HUVEC monolayer permeability and suggested a role for HUVEC $[Ca^{++}]_i$ in regulating the integrity of junctions between EC. FMLP stimulated PMN induced increases in HUVEC $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ (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 $[Ca^{++}]_i$ and

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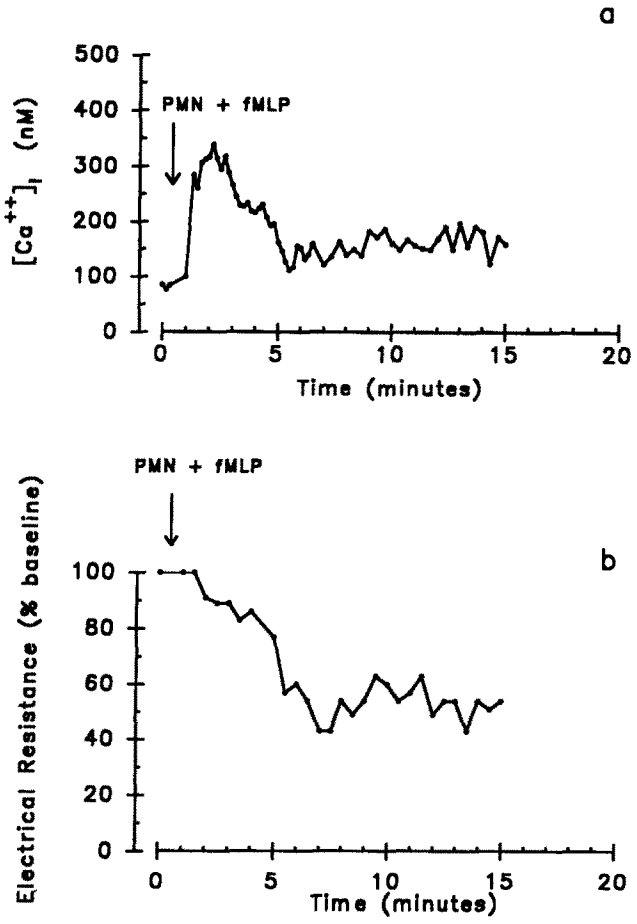

Figure 4. HUVEC $(Ca^{++})_i$ and transendothelial electrical resistance in response to the interaction of fMLP stimulated PMN with HUVEC. A representative curve is shown of (*a*) $[Ca^{++}]_i$ in a single HUVEC (n = 4) and (*b*) the electrical resistance across a HUVEC monolayer (n = 5) before and after the addition of PMN (PMN:HUVEC ratio = 10:1) and fMLP (10^{-7} M) to the apical surface of the HUVEC monolayer. 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. Experiments were performed in the presence of HBS/HIFBS or HBS/BSA.

the rate or extent of the decrease in transendothelial electrical resistance. This is not surprising, because changes in $[Ca^{++}]_i$ are monitored in single EC whereas changes in electrical resistance reflect the permeability of an entire EC monolayer. What is clear is that the $[Ca^{++}]_i$ changes observed in a single HUVEC are representative of most of the HUVEC in a monolayer and that agents that cause two to eightfold fold increases in HUVEC $[Ca^{++}]_i$ promote decreases in transendothelial resistance.

Polymorphonuclear Leukocyte Migration across Human Umbilical Vein Endothelial Cell Monolayers in Response to IL-1 Treated Human Umbilical Vein Endothelial Cell Monolayers Induces a Five to Sixfold Transient Increase in Human Umbilical Vein Endothelial Cell Cytosolic Free Calcium

PMN migration across an endothelium can occur by at least two distinct mechanisms: a PMN initiated mechanism in which PMN respond to soluble chemoattractants and an EC initiated mechanism in which EC monolayers treated with

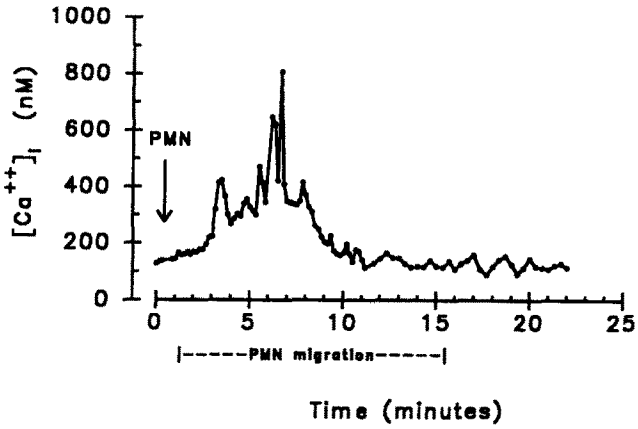

Figure 5. HUVEC $[Ca^{++}]_i$ during PMN migration across an IL-1 treated HUVEC monolayer (n = 5). HUVEC monolayers were incubated with recombinant IL-1 β (5 U/ml) for 4 h at 37°C, washed, and loaded with fura-2. $[Ca^{++}]_i$ in a single HUVEC was measured in the presence of HBS/BSA and after the addition of PMN (PMN:HUVEC ratio = 10:1) to the apical surface of HUVEC monolayers in the absence of any other chemoattractant or IL-1. PMN migration across the HUVEC monolayer occurred during the interval indicated on the horizontal axis. 2.4 ± 0.6 PMN for each HUVEC migrated under these conditions.

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 $[Ca^{++}]_i$. 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ 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

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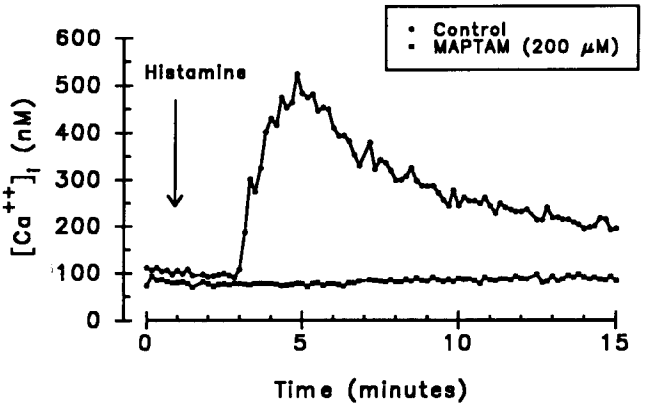

Figure 6. Effect of MAPTAM on histamine induced increases in HUVEC $[Ca^{++}]_i$ (n = 3). HUVEC monolayers were incubated with HBS/FBS (2%) with or without MAPTAM (200 μM) for 30 min at 37°C, washed, then loaded with fura-2. Representative curves are shown of $[Ca^{++}]_i$ in the presence of HBS/BSA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

HUVEC $[Ca^{++}]_i$ play a role in transendothelial PMN migration, we examined the effect of clamping HUVEC $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ induced by fMLP-treated PMN. HUVEC $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ in MAPTAM treated cells, fura-2 fluorescence was measured in the presence of saturating and minimum $[Ca^{++}]_i$ and $MnCl_2$ 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 further incubated to allow hydrolysis of intracellular MAPTAM as described above for $[Ca^{++}]_i$ 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

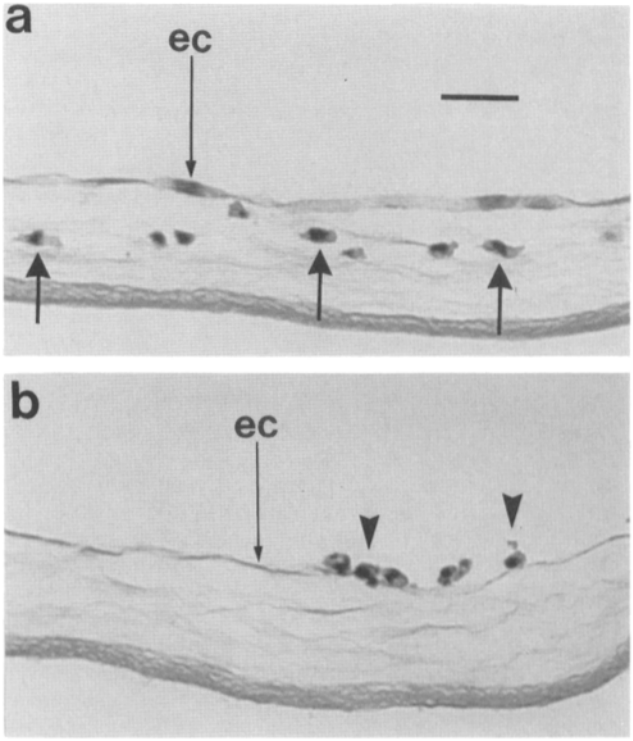

Figure 7. Cross-sections of MAPTAM treated or control HUVEC monolayers incubated with fMLP-stimulated PMN. HUVEC monolayers were incubated with or without MAPTAM as described under Materials and Methods. PMN were added to the apical surface of (*a*) control or (*b*) MAPTAM-treated HUVEC monolayers and allowed to migrate toward fMLP on the opposite side of the monolayer for 30 min. Cultures were fixed and sectioned. ≥80% of PMN (*arrows*) associated with control monolayers in contrast to ≤21% of PMN associated with MAPTAM-treated monolayers migrated across the HUVEC monolayer (*ec*). ≥79% of PMN associated with MAPTAM treated monolayers were adherent to the apical surface of HUVEC monolayers (*arrowheads*). Bar, 25 μm.

monolayer was quantified as described in Materials and Methods. In response to an fMLP gradient, 58 ± 4 PMN/400× 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/400× 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*).

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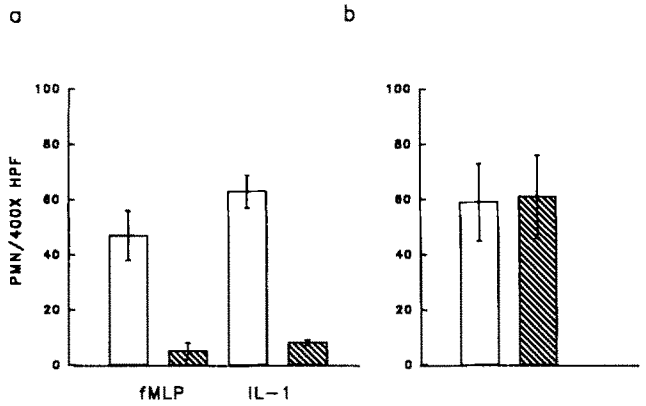

Figure 8. Effect of MAPTAM on PMN interactions with HUVEC. HUVEC monolayers were incubated with or without MAPTAM as described under Materials and Methods. 2×10^6 PMN (PMN:EC of 5:1) were added to the apical surface of HUVEC monolayers. (*a*) PMN migration was stimulated by adding fMLP below HUVEC monolayers ($n = 3$) or by treating HUVEC monolayers with IL-1 ($n = 3$). (*b*) PMN-EC adhesion was stimulated by adding fMLP together with PMN above HUVEC monolayers ($n = 2$). 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 $[Ca^{++}]_i$ 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 monolayers 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 $[Ca^{++}]_i$ affect transendothelial PMN migration by opening junctions between HUVEC, we examined the effect of clamping HUVEC $[Ca^{++}]_i$ 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 $13 \pm 2\ \Omega \cdot cm^2$. 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 $22 \pm 6\%$ decrease in the resistance of control monolayers ($n = 3$) but only a $8 \pm 4\%$ decrease in the resistance of MAPTAM treated monolayers ($n = 3$). Thus clamping HUVEC $[Ca^{++}]_i$ at resting levels significantly reduced PMN induced increases in HUVEC monolayer permeability to ions. These findings indicate that by promoting increases in HUVEC $[Ca^{++}]_i$, 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 $[Ca^{++}]_i$. Second, they show that this rise in $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$, 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$

We selected EC $(Ca^{++})_i$ as an indicator of trans-cellular signaling between PMN and EC because of the general importance of $[Ca^{++}]_i$ as a second messenger and because of its involvement in cytoskeletal functions. A rise in EC $[Ca^{++}]_i$ 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

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unstimulated PMN across monolayers of IL-1 treated EC, induces an increase in EC $[Ca^{++}]_i$ 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 B_4 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 ($\leqslant$150 Å). 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 $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ in Transendothelial Migration of Stimulated Polymorphonuclear Leukocytes

PMN require physiological concentrations of Ca^{++} in the medium in order to bind to their cognate ligands on EC (16). Similarly, physiological concentrations of 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 Ca^{++}-free solutions. Therefore we used a cell-permeant Ca^{++} chelator (MAPTAM) to clamp $[Ca^{++}]_i$ in EC at resting levels ($\sim$100 nM), while maintaining these cells in Ca^{++} replete (1 mM) medium. Experiments showed that such MAPTAM-treated EC exhibited no increase in $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ 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 Ca^{++} chelator before addition of the PMN. More importantly, Kuijpers et al. (22) showed that buffering of $[Ca^{++}]_i$ 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 $[Ca^{++}]_i$ is necessary for PMN to migrate across endothelia.

A Unifying Hypothesis

How might changes in EC $[Ca^{++}]_i$ 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 Ca^{++} and calmodulin dependent (1). Activation of EC myosin causes these cells to retract (34). EC $[Ca^{++}]_i$ 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.

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