Roles of Calcium, Cyclic Nucleotides, and Protein Kinase C in Regulation of Endothelial Permeability

Yoshiji Yamada, Takeo Furumichi, Hirohiko Furui, Toyoharu Yokoi, Takahiko Ito, Kazunobu Yamauchi, Mitsuhito Yokota, Hiroshi Hayashi, and Hidehiko Saito

We studied the effects of calcium, cyclic nucleotides, and protein kinase C on albumin transfer, electrical resistance, and cytoskeletal actin filaments in cultured human umbilical vein endothelial cells. The endothelial monolayer grown on collagen-treated filters markedly restricted the transfer of albumin relative to its transfer across the filter alone. Both Ca++ ionophore A23187 and ethyleneglycol tetraacetlc acid disrupted the integrity of the endothelial monolayer, thereby increasing endothelial albumin transfer and decreasing electrical resistance in a concentration-dependent manner. Neither W-7, a calmodulin antagonist, nor TMB-8, an intracellular Ca++ antagonist, influenced endothelial permeability. In contrast, increases in intracellular cyclic adenosine 5’-monophosphate (AMP) and/or cyclic guanosine 5’-monophosphate (GMP) induced by dibutyl cyclic AMP, forskolin, 3-isobutyl-1-methyoxanthine, 8-bromo cyclic GMP, dibutyryl cyclic GMP, or sodium nitroprusside significantly elevated endothelial electrical resistance and inhibited albumin transfer; similar effects resulted from activation of protein kinase C by phorbol-12-myristate-13-acetate or 1-oleoyl-2-acetyl-glycerol. These substances ruffled the dense peripheral bands of F-actin without compromising the integrity of endothelial monolayer. These results suggest that calcium, cyclic nucleotides, and protein kinase C play important roles in the regulation of endothelial permeability and the maintenance of endothelial integrity. (Arteriosclerosis 10:410-420, May/June 1990)

Vascular endothelial cells (ECs) constitute the inner lining of the vascular wall and act as a selective barrier to macromolecules in the blood. Recent studies suggest that the pathogenesis of atherosclerosis involves endothelial damage, which reduces the effectiveness of these cells as a barrier.

The development of EC culture has allowed the study of permeability in EC monolayers in porous-bottom dishes. Such in vitro models have the advantage over in vivo studies of permitting direct measurement of EC permeability. Transendothelial movements of several macromolecules of different molecular weights and radii have been investigated. We chose to study albumin because it is one of the most important physiologic macromolecules, determining the balance of osmotic pressure across the endothelium and acting as a barrier to a wide variety of cellular functions. There have been several studies concerning the effects of calcium and cyclic adenosine 5’-monophosphate (AMP) on endothelial permeability. Shasby and Shasby reported that extracellular calcium depletion caused retraction of porcine pulmonary artery ECs, resulting in an increase in albumin transfer and a decrease in electrical resistance. They also reported that the Ca++ ionophore A23187 increased endothelial albumin transfer and mimicked oxidant-induced changes in cell shape. Stelzner et al. recently showed that the barrier function of bovine pulmonary artery EC monolayer was enhanced by an increase in intracellular cyclic AMP. Mizuno-Yagyu et al. observed that prostacyclin and its stable derivatives inhibited fluorescein dextran transport of porcine arterial ECs by increasing cyclic AMP production. However, the effects of calmodulin antagonist, intracellular Ca++ antagonist, and cyclic guanosine 5’-monophosphate (GMP) on EC permeability have not yet been understood.

Furthermore, there has been little investigation of the consequences of these cytosolic events in terms of the permeability of cultured human umbilical vein ECs, even though EC permeability appears to vary among species and at different vascular sites.

Protein kinase C plays a crucial role in cellular signal transduction. Activation of protein kinase C by phorbol esters enhances tight junction permeability in monolayers derived from renal epithelial cell lines. It has also been reported that the permeability of pulmonary artery ECs was increased by the stimulation of protein kinase C by phorbol-12,13-dibutyrate, hydrogen...
peroxide,\textsuperscript{18} or thrombin.\textsuperscript{19} However, the role of this enzyme in the regulation of endothelial permeability is poorly understood.

In the present study, we investigated the effects of calcium, cyclic nucleotides, and protein kinase C on albumin transfer and electrical resistance in human umbilical vein ECs cultured on collagen-treated filters. We also attempted to determine whether or not the permeability changes induced by these substances paralleled the morphological alterations of ECs.

\section*{Methods}

\section*{Materials}

The following materials were used: Transwell-COL (6.5 mm diameter, 0.4 \textmu m pore size, collagen-treated membrane); 24-well culture plates (Costar, Cambridge, MA); plastic tissue culture flasks, pipets, and centrifuge tubes (Becton Dickinson, Lincoln Park, NJ); fetal bovine serum (FBS) (General Scientific Laboratories, Los Angeles, CA); MCDB 104 medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan); G1T medium (Nihon Pharmaceutical, Tokyo, Japan); trypsin (GIBCO Laboratories, Grand Island, NY); endothelial cell growth supplement (ECGS); epidermal growth factor (EGF) (Collaborative Research, Bedford, MA); human serum albumin (HSA, fraction V, fatty acid-free) (Miles Laboratories, Kankakee, IL); penicillin G and streptomycin (Meiji Seika, Tokyo, Japan); porcine skin gelatin, porcine intestinal heparin, Ca\textsuperscript{2+} ionophore A23187, ethyleneglycol-bis-(-\textbeta-aminohexyl ether) N,N',N',N'-tetraacetic acid (EGTA), N-(6-aminoheptyl)-5-chloro-1-naphthalenesulfonamide (W-7), N-(6-aminoheptyl)-1-naphthalenesulfonamide (W-5), 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8), \(3\textbeta,5\textalpha\)-O-dibutyryladenosine 3':5'-cyclic monophosphate (db cAMP), \(\textbeta\)-acetoxy-8,13-epoxy-1\textalpha,6\textbeta,9\textepsilon-hydroxy-14-one-11-one (forskolin), 3-isobutyl-1-methylxanthine (IMX), 8-bromoguanosine 3':5'-cyclic monophosphate (8-br cGMP), \(3\textbeta,5\textalpha\)-O-dibutyrylguanosine 3':5'-cyclic monophosphate (db cGMP), sodium nitroprusside, phorbol-12-myristate-13-acetate (PMA), 1-octyl-2-acetyl-glycerol (OAG), and 4\textalpha-phorbol 12,13-didecanoate (4\textalpha-PDD) (Sigma Chemical, St. Louis, MO); ethylenediaminetetraacetic acid (EDTA) (Katayama Chemical, Osaka, Japan); Vitrogen 100 (Collagen, Palo Alto, CA); Lab-Tek tissue culture chamber slides (Miles Laboratories); rhodamine phalloidin (Molecular Probes, Eugene, OR); monoclonal mouse anti-von Willebrand factor antibody (Dako, Santa Barbara, CA); fluorescein-conjugated Fab fragment goat anti-mouse IgG (Tago, Burlingame, CA); and an epithelial voltohmmeter (EVOM) (World Precision Instruments, New Haven, CT).

\section*{Cell Culture}

ECs were obtained from human umbilical veins according to the method of Jaffe et al.\textsuperscript{20} with minor modifications. The umbilical vein was cannulated at both ends and was filled with Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free phosphate-buffered saline (PBS) containing 0.1% trypsin and 0.02% EDTA; this was incubated at room temperature for 15 minutes. The cell suspension was centrifuged at 130 g for 5 minutes at 4°C and was resuspended in the complete culture medium (MCDB 104 supplemented with 100 U/ml penicillin G, 100 \mu g/ml streptomycin, 75 \mu g/ml ECGS, 10 ng/ml EGF, 100 \mu g/ml heparin, and 10% FBS). The suspended cells were seeded into 25 cm\textsuperscript{2} flasks precoated with 0.1% gelatin and were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2}. The medium was changed 1 day after the culture was started and twice a week thereafter. The cells were usually confluent within 7 to 14 days, at which time their passage followed a brief exposure to 0.025% trypsin. The cells were identified as ECs by their morphological characteristics,\textsuperscript{20} evidence of angiotensin converting enzyme activity,\textsuperscript{21} and indirect staining with FITC-labeled von Willebrand factor antibody.\textsuperscript{22}

\section*{Plating of Endothelial Cells on Micropore Filters}

The filters of the upper chambers were coated with type I collagen (Vitrogen 100) diluted to 1:10 with MCDB 104 at 37°C for 45 minutes. All experimental data were obtained from ECs of the 2nd to 4th passage, which were harvested when they reached confluence. After brief trypsinization, the cell suspension was centrifuged at 130 g for 5 minutes at 4°C. The cell pellet was resuspended in the complete culture medium and was plated on a collagen-treated micropore filter at a density of 5 \times 10\textsuperscript{5} cells/cm\textsuperscript{2} (Figure 1). The cells were then incubated and were used for experimentation 1 day after plating.

\section*{Endothelial Albumin Transfer}

The culture media in the upper and lower chambers were aspirated, and both chambers were washed once with GIT medium. Then 0.15 ml of GIT containing 10 mg/ml HSA was placed in each upper chamber and 0.7 ml GIT without HSA in each lower chamber. To avoid
Endothelial Electrical Resistance

Endothelial electrical resistance was measured by an EVOM with stick electrodes. The arm of each electrode has at its tip a central Ag/AgCl voltage sensor electrode and a concentric annular silver band for passing current through the EC monolayer. One electrode was dipped into the medium at the center of the upper chamber, and the second electrode, into the medium of the lower chamber (Figure 1). Electrical resistance was determined by passage of a 20 μA DC current through the EC monolayer and measurement of the resulting voltage gradient across it. The resistance of the endothelial monolayer was calculated as the total resistance (that of the filter, medium, and ECs) minus the background resistance (that of the filter and medium).

### Results

**Morphology of Endothelial Cells**

A light photomicrograph of a confluent monolayer of ECs on a collagen-treated micropore filter stained with silver nitrate is shown in Figure 2. The cell’s contour was demonstrated by silver deposits in intercellular junctions. The ECs were confluent 1 day after plating, and no

<p>| Table 1. Calculated Free Ca++ Concentration in GIT |
|-------------------------------|-----------------------------------------------|</p>
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All values are expressed in mM. Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) = EGTA concentration added to GIT; Ca++-free = calculated free Ca++ concentration in GIT.

the effect of hydrostatic pressure across the monolayer, the fluid levels in the upper and lower chambers were kept equal. Before each experiment, the ECs were carefully examined by phase-contrast microscopy. If an endothelial monolayer had intracellular gaps, it was discarded. Furthermore, the EC monolayer was confirmed to be confluent by a silver nitrate staining.

**EGTA ionophore A23187** (10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M), EGTA (0.1, 0.25, 0.5, 1.0, or 2.0 mM), W-7 (10^{-6}, 2x10^{-6}, or 5x10^{-6} M), W-5 (10^{-6}, 2x10^{-6}, or 5x10^{-6} M), or TMB-8 (10^{-6}, 2x10^{-6}, or 5x10^{-6} M) was added to the EC monolayer for an investigation of the effect of calcium on endothelial albumin transfer. A concentration of A23187 higher than 10^{-5} M and that of EGTA higher than 2 mM had cytotoxic effects on ECs. Therefore, 10^{-8} to 10^{-5} M A23187 and 0.1 to 2.0 mM EGTA were used in the experiments. The addition of W-7, W-5, or TMB-8 higher than 10^{-5} M was also found to cause lactate dehydrogenase (LDH) release from ECs, suggesting that these concentrations of the agents had cytotoxic effects on ECs. Therefore, we used these agents in a concentration of 10^{-8} to 5x10^{-6} M. The degree of calcium chelation with EGTA was estimated from the theoretical free Ca++ concentration in GIT (Table 1). For study of the roles of cyclic nucleotides, db cAMP (10^{-4} or 10^{-3} M), forskolin (10^{-6} or 10^{-5} M), IMX (5x10^{-6} or 5x10^{-5} M), 8-br cGMP (10^{-4} or 10^{-3} M), db cGMP (10^{-4} or 10^{-3} M), or sodium nitroprusside (10^{-6} or 10^{-5} M) was added to the EC monolayer. The influence of protein kinase C was examined by the addition of PMA (10^{-6} or 10^{-7} M), OAG (10^{-5} or 10^{-4} M), or 4α-PDD (10^{-7} M) to the EC monolayer. In preliminary experiments, we examined whether or not the effects were different when the agents were added to the upper chamber alone or to both upper and lower chambers. The results were not significantly different. Therefore, in the present study, each agent was added to the upper chamber alone. The EC monolayer-chamber system was incubated for 60 minutes, after which the media from the lower chambers were aspirated and kept at −30°C. The albumin concentration was measured by a radioimmunoassay (Pharmacia AB, Uppsala, Sweden).

**Statistical Analysis**

All data are expressed as means±SEM. In the studies of albumin transfer and electrical resistance, the data were subjected to one-way analysis of variance and Scheffe’s multiple-range test. Differences were considered significant if p<0.05.

**Morphological Analysis**

ECs cultured on the collagen-treated filters were stained with silver nitrate.6 The cells were washed with 5% dextrose and treated with 0.4% silver nitrate for 20 minutes. Then the ECs were washed with 5% dextrose and treated with a 1:1 mixture of 3% cobalt bromide and 1% ammonium bromide for 5 minutes. After washing with 5% dextrose, the filter was cut from the plastic part of the chamber and mounted.

For investigation of endothelial actin filaments, ECs were cultured in Lab-Tek tissue culture chamber slides and stained with rhodamine phalloidin, which is specific for F-actin.24 The cells were briefly rinsed with PBS and fixed in 3.0% formaldehyde in PBS for 20 minutes. They were rinsed with PBS and permeabilized with 0.1% Triton X-100 for 4 minutes. After washing with PBS, they were incubated with 3.3x10^{-6} M rhodamine phalloidin in PBS for 30 minutes. The cells were then rinsed in PBS and mounted over glycerol/PBS (1:1).

For phase-contrast and light microscopy, the specimens were viewed with an Olympus IMT-2 and were photographed with Fuji Neopan F film (ASA 32). For fluorescence microscopy, the specimens were examined under an Olympus BH-2 and were photographed with Fujichrome DX400D film (ASA 400).

The endothelial monolayer was further examined by electron microscopy. The filter with the adherent EC monolayer was fixed in 3% formaldehyde in PBS for 20 minutes, then fixed in 3.0% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4). It was postfixed in 1% osmium tetroxide, dehydrated in an ascending ethanol series, and embedded in an EPON 812 mixture. After ultrathin sectioning, the specimens were stained with uranium acetate and lead citrate and were examined with a Hitachi H-600 electron microscope.
Figure 2. A light photomicrograph of a confluent monolayer of endothelial cells (ECs) on a collagen-treated micropore filter stained with silver nitrate. The ECs exhibit a cobblestone pattern 1 day after plating on the filter. No intercellular gaps are present. Bar=40 μm.

Figure 3. A cross-sectional transmission electron micrograph of a confluent endothelial monolayer on the filter, showing electron-dense junctional structures (arrows). Bar=0.2 nm.

intercellular gap formation was observed. A cross-sectional transmission electron microscopic examination demonstrated electron-dense structures in focal zones of intercellular junctions (Figure 3).

Endothelial Albumin Transfer and Electrical Resistance

ECs grown on collagen-coated filters markedly restricted the transfer of albumin (41.1±2.0 μg/ml, n=10), as compared to its transfer across the filters alone (388.0±17.1 μg/ml, n=10, p<0.001, nonpaired t test) after 60 minutes of incubation. ECs also impeded the flow of electrical current. The electrical resistance of the confluent endothelial monolayer was 9.6±0.6 Ω·cm² (n=10).

The addition of Ca²⁺ ionophore A23187 to the EC monolayer increased the albumin transfer and decreased electrical resistance in a concentration-dependent manner (Figure 4A). A23187 (10⁻⁵ M) increased albumin transfer from 42.3±2.6 to 95.0±11.5 μg/ml (n=10) and decreased electrical resistance from 9.7±0.7 to 3.0±0.6 Ω·cm² (n=6). Chelation of extracellular calcium with EGTA also promoted albumin transfer and reduced electrical resistance in a dose-dependent manner (Figure 4B). EGTA (2.0 mM) enhanced albumin transfer from 46.7±4.9 to 129.8±4.9 μg/ml (n=6) and lowered electrical resistance from 9.5±0.2 to 2.6±0.5 Ω·cm² (n=9). However, W-7, a calmodulin antagonist; W-5, a control drug of W-7; and TMB-8, an intracellular Ca²⁺ antagonist, had no effect on endothelial albumin transfer or electrical resistance (Figures 5A to 5C).

In contrast, compounds capable of increasing intracellular cAMP and/or cGMP concentrations reduced the permeability of ECs (Figures 6A to 6F). The addition of 10⁻³ M dibutyryl cAMP lowered albumin transfer from 10.2±0.4 to 2.9±0.5 μg/ml (n=7) and increased electrical resistance from 9.9±0.4 to 15.4±1.8 Ω·cm² (n=6, Figure 6A). Forskolin (10⁻⁵ M), an activator of adenylate cyclase, decreased the transport of albumin from 40.4±4.1 to 10.2±0.4 μg/ml (n=7) and raised electrical resistance from 9.9±0.4 to 15.4±1.8 Ω·cm² (n=6, Figure 6B). IMX (5×10⁻⁴ M), an inhibitor of cyclic nucleotide phosphodiesterase, also decreased endothelial albumin transfer from 42.2±2.5 to 12.6±1.3 μg/ml (n=6) and increased electrical resistance from 9.8±0.4 to 15.9±0.4 Ω·cm² (n=6, Figure 6C). When ECs were treated with 10⁻³ M
ARTERIOSCLEROSIS VOL 10, NO 3, MAY/JUNE 1990

Figure 5. The effects of W-7 (A), W-5 (B), and TMB-8 (C) on endothelial albumin transfer (open bars) and electrical resistance (striped bars). W-7, W-5, and TMB-8 had no significant effect on endothelial albumin transfer or electrical resistance. See the Methods section for an explanation of the abbreviations.

8-br cGMP, albumin transfer decreased from 44.5±3.3 to 15.8±2.5 μg/ml (n=8), and electrical resistance increased from 9.6±0.2 to 15.1±0.9 Ω·cm² (n=6, Figure 6D). The addition of 10⁻³ M db cGMP reduced endothelial albumin transfer from 41.2±6.2 to 21.3±1.9 μg/ml (n=6) and enhanced electrical resistance from 9.6±0.3 to 14.9±0.9 Ω·cm² (n=6, Figure 6E). Sodium nitroprusside (10⁻⁵ M), a guanylate cyclase activator, also inhibited endothelial albumin transfer from 40.1±5.3 to 19.3±3.2 μg/ml (n=7) and elevated electrical resistance from 9.6±0.4 to 15.2±0.9 Ω·cm² (n=6, Figure 6F).

Activation of protein kinase C by PMA or OAG apparently inhibited endothelial permeability (Figures 7A, 7B, 7D, and 7E). When the EC monolayers cultured with ECGS and EGF were exposed to 10⁻⁷ M of PMA, a tumor-promoting phorbol ester, albumin transfer decreased from 46.3±3.6 to 10.4±1.2 μg/ml (n=8) and electrical resistance increased from 9.5±0.5 to 14.9±0.8 Ω·cm² (n=6, Figure 7A). When ECs were cultured on the filters without ECGS or EGF, PMA (10⁻⁷ M) also inhibited the transport of albumin from 54.8±2.9 to 16.8±2.2 μg/ml (n=5) and enhanced electrical resistance from 8.0±0.3 to 13.9±0.4 Ω·cm² (n=5, Figure 7D). The addition of 10⁻⁴ M of OAG, a synthetic diacylglycerol, to EC monolayers cultured with ECGS and EGF reduced albumin transfer from 47.8±6.4 to 20.5±1.1 μg/ml (n=8) and increased electrical resistance from 9.9±0.5 to 15.0±0.5 Ω·cm² (n=6, Figure 7B). OAG (10⁻⁴ M) also lowered albumin transfer from 53.0±5.1 to 27.4±3.8 μg/ml (n=5) and elevated electrical resistance from 8.1±0.3 to 13.1±0.5 Ω·cm² (n=5) in ECs cultured without ECGS or EGF (Figure 7E). On the other hand, 4α-PDD (10⁻⁷ M), a PMA analogue that does not activate protein kinase C, had no effect on endothelial albumin transfer or electrical resistance (Figures 7C and 7F). The permeability of ECs cultured without ECGS or EGF was relatively higher than that of ECs cultured with ECGS and EGF.

Morphological Analysis

A phase-contrast photomicrograph of the control EC monolayer showed that ECs appeared as essentially homogenous populations of uniformly sized, tightly packed polygonal or spindle cells and that there was no intercellular gap formation (Figure 8A). The addition of A23187 caused contraction of ECs and consequent intercellular gap formation (Figure 8B). Chelation of extracellular calcium with EGTA caused retraction of ECs, resulting in loss of integrity of the endothelial monolayer (Figure 8C). In contrast, W-7, W-5, and TMB-8 did not significantly influence EC shape or integrity.

The rhodamine phalloidin-stained actin filaments in the control ECs were arranged as linear stress fibers and dense peripheral bands (Figure 9A). The dense peripheral bands of F-actin of adjacent cells were closely apposed to one another, and there was no intercellular gap formation (Figure 9A). To examine EC junctions more directly, we attempted to stain the ECs with anti-plakoglobin antibody (Progen Biotechnik, Heidelberg, FRG). However, EC junctions were not clearly demonstrated with this antibody. The addition of db cAMP, forskolin (Figure 9B), IMX, 8-br cGMP (Figure 9C), db cGMP, or sodium nitroprusside similarly affected the F-actin. The dense peripheral band of F-actin was ruffled, giving the ECs an irregular and complex contour. However, endothelial integrity was maintained, and no intercellular gaps formed. The effects of PMA (Figure 9D) and OAG on F-actin were similar to those of the compounds that increase cAMP and/or cGMP; the dense peripheral bands of F-actin were ruffled, but EC integrity was maintained. In contrast, 4α-PDD had no effect on F-actin.

Discussion

The regulation of vascular permeability is one of the most important functions of ECs; interference with this function
may promote pathological changes in the vascular wall. We investigated the permeability characteristics of human umbilical vein ECs cultured on a collagen-treated micropore filter. We chose albumin for our experimental model of the transport of high molecular weight substances across the EC monolayer. In addition, we measured endothelial electrical resistance, which is a sensitive, rapid, and noninvasive method of determining ion permeability. The in vitro system described here is a simple model of microvasculature in which the cellular regulatory mechanisms of endothelial permeability can be directly investigated.

Measurement of endothelial albumin transfer and electrical resistance requires that the EC monolayer be intact and confluent. As shown in Figure 2, we verified this to be the case by means of a silver staining procedure. Cross-sectional transmission electron microscopy demonstrated that our in vitro EC monolayer closely resembled in vivo vascular endothelium in terms of intercellular junctional structures (Figure 3).

The amount of albumin transported through the EC monolayer and filter was much smaller than that transported through the filter alone, which indicates that the cultured endothelial monolayer functioned as a barrier to albumin. The electrical resistance of our EC monolayer was 9.6±0.6 Ω·cm², which is in good agreement with previously reported results. In this study, changes in...
Figure 7. The effects of PMA (A,D), OAG (B,E), and 4α-PDD (C,F) on albumin transfer (open bars) and electrical resistance (striped bars) in EC monolayers cultured on the filters with ECGS and EGF (A to C) or without ECGS or EGF (D to F). Both PMA and OAG elevated endothelial electrical resistance and inhibited albumin transfer, whereas 4α-PDD affected neither parameter. *p<0.05, **p<0.01 relative to the control value (A,B, Scheffe’s multiple-range test; C to F, nonpaired t test). See the Methods section for an explanation of the abbreviations.

electrical resistance of EC monolayer were inversely proportionate to the amount of albumin transported. These results indicate that electrical resistance can be a sensitive indicator of changes in EC permeability.

Different types of endothelial and epithelial cells have so far been used for investigating permeability characteristics. Table 2 represents a detailed comparison of our EC monolayer to others.456825 -29 The electrical resistances of bovine adrenal microvascular ECs (69 Ω·cm²)26 and MDCK cells (181 Ω·cm²)28 are much higher than that of our human endothelial monolayer. Albumin permeability of our EC monolayer appeared to be lower than that of bovine arterial ECs reported by other investigators. However, our result is in good agreement with that of Huang et al.,28 who used human umbilical vein ECs.

Many chemical substances have been reported to increase endothelial albumin transfer, including thrombin,29 histamine,30 platelet activating factor,31 and oxygen radicals.7 The permeability changes induced by these substances may be mediated by cellular signal transduction.7,30 Because calcium, cyclic nucleotides, and protein kinase C have important roles in cytosolic signaling events, we attempted to elucidate the effects of these substances on the regulation of EC permeability.
Figure 8. Phase-contrast photomicrographs in a control EC monolayer (A) and in ECs exposed to 10^{-5} M of A23187 (B) and 2 mM of EGTA (C). Gap = intercellular gap formation. Bar = 40 μm.

Although they may interact with one another in signal-transducing systems,^{13,14} we investigated these substances separately to avoid confusion.

A23187 and EGTA disrupted the integrity of the endothelial monolayer, thereby increasing albumin transfer and decreasing electrical resistance. Changes in EC shape and formation of intercellular gaps after treatment with these agents were demonstrated by a phase-contrast microscopy. Olesen^{10} reported that Ca^{2+} ionophores increased endothelial ion permeability in frog brain venules, as reflected by reduced electrical resistance. Our observation that A23187 exerts a direct effect on cultured human ECs is similar to his finding. The effects of A23187 and EGTA on the permeability of the cultured human umbilical vein ECs that we studied appear to be similar to those on porcine pulmonary artery ECs reported by Shasby et al.^{6,7} Calcium is also important in the regulation of the cytoskeletal assembly, which is critical to the definition of cell shape.^{32,33} Therefore, disturbances of intra- and extracellular calcium homeostasis can produce changes in both EC shape and permeability. In the present study, neither inhibition of calmodulin by W-711 nor stabilization of Ca^{2+} binding to Ca^{2+} storage site by TMB-8 influenced EC permeability.

The increase in intracellular cAMP and/or cGMP elevated electrical resistance and suppressed albumin transfer in cultured human umbilical vein ECs. Our data on the effect of cAMP on the permeability of human umbilical vein ECs are in good agreement with those in a recent report by Stezner et al.,^{8} who used bovine pulmonary artery ECs. Duffey et al.^{34} showed that cAMP reduced the ion permeability of the paracellular pathway in gall bladder epithelium by producing tight junctions. It is possible that the reduction of endothelial permeability by cAMP is due in part to an increase in the number of tight junctions. In our study, 8-bromo cGMP, db cGMP, and sodium nitroprusside also inhibited endothelial albumin transfer and elevated electrical resistance. These results indicate that not only cAMP, but also cGMP, contribute to the regulation of EC permeability.

Protein kinase C plays a crucial role in the transduction of extracellular stimuli for activation of cellular function.^{13,14} PMA and OAG activate protein kinase C, substituting for the endogenous messenger, diacylglycerol, one of the products of phospholipid breakdown.^{35,36} Our results demonstrate that activation of protein kinase C inhibits endothelial albumin transfer and increases electrical resistance in the presence or absence of ECGS and EGF. Ojakian^{19} and Mullin and O'Brien^{10} reported that tumor-promoting phorbol esters increased the permeability of renal epithelial cell lines. The stimulation of protein kinase C also enhanced the permeability of pulmonary artery ECs.^{17,18,19} The differences between their results and ours may be attributable to differences in permeability characteristics among the renal epithelial cell lines (MDCK^{15} and LLC-PK, cells^{16}), pulmonary artery ECs,^{17,18,19} and human umbilical vein ECs. Therefore, the observations in this study may be relevant to only limited vascular (human umbilical vein) ECs.

A fluorescence microscopic study for F-actin showed that the dense peripheral bands of F-actin were ruffled by the addition of agents that increase intracellular cAMP and/or cGMP or activate protein kinase C. However, adjacent ECs did not break contact, and the integrity of EC monolayer was maintained. These results indicate that these substances affect cytoskeletal F-actin of ECs. In addition, cAMP- and cGMP-dependent protein kinases...
Figure 9. F-actin stained with rhodamine phalloidin in a control EC monolayer (A) and in ECs exposed to $10^{-5}$ M of forskolin (B), $10^{-3}$ M of 8-br cGMP (C), and $10^{-7}$ M of PMA (D). DPB = dense peripheral band, SF = stress fiber. Bar = 20 μm. See the Methods section for an explanation of the abbreviations.

Table 2. Comparisons of Albumin Permeability and Electrical Resistance from Various Endothelial and Epithelial Monolayers

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</tbody>
</table>

Albumin permeability is expressed as a permeability coefficient for albumin. ECs = endothelial cells.
and protein kinase C phosphorylate various cellular proteins, including cytoskeleton-associated proteins. Therefore, the increase in cyclic nucleotides and the activation of protein kinase C may lower EC permeability, at least in part, through effects on the EC cytoskeleton.

Recent studies indicate that fluorescence staining for plakoglobin, an intercellular junctional plaque protein, directly demonstrates intercellular junction of the EC monolayer. However, we could not clearly demonstrate staining of plakoglobin in EC junctions. The reason for failure to stain endothelial plakoglobin is not clear. The antibody we have used is raised against bovine snout epidermal desmosomes. Interestingly, Franke et al. also pointed out that the reaction of similar antibodies with mammalian endothelial junctions is very weak.

In conclusion, the results of our study indicate that calcium, cyclic nucleotides, and protein kinase C play important roles in the regulation of permeability and the maintenance of integrity in cultured human umbilical vein ECs. Because the permeability characteristics of ECs vary among species and at different vascular sites, these findings should be strictly applied to this kind of cell. It is expected that future investigation will elucidate the mechanisms of those regulatory events at the molecular level.

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