Vacular-type $H^+$-ATPases at the plasma membrane regulate pH and cell migration in microvascular endothelial cells


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Microvascular endothelial cells are uniquely positioned within vessels of the macro- and microcirculation. Macro- and microvascular endothelial cells play an important role in regulating blood vessel tone and blood flow by synthesizing and secreting paracrine and autocrine growth factors and hormones (10, 15). Endothelial cells also secrete proteolytic enzymes, which are needed for formation of new capillary networks, a necessary step in vascular remodeling (13).

Changes in intracellular (cytosolic) pH ($p_{\text{Hcyt}}$) are important in signal transduction mechanisms, which regulate many physiological processes, including cell growth, secretion, contraction, and invasion/migration (18, 44, 47). These processes are important in angiogenesis and vascular remodeling (13). The regulation of $p_{\text{Hcyt}}$ in most eukaryotic cells, including endothelial cells, is mediated by the $Na^+/H^+$ exchanger and $HCO_3^-$dependent $H^+$-transporting mechanisms (11, 17, 22, 68). In some specialized and highly invasive cells (metastatic cells, macrophages, neutrophils, and osteoclasts), plasma membrane vacuolar (V) $H^+$-ATPases (pmV-ATPases) are also used to regulate $p_{\text{Hcyt}}$ (27, 35, 50, 58). These ATPases are distinguished from other proton pumps by their pharmacological inhibition (8, 33). The V-ATPases are inhibited by bafilomycin A1, concanamycin, salicylihalamide, and 7-chloro-4-dinitrobenz-2-oxa-1,3-diazole, which have no effect on the P- or F-type ATPases (6, 9, 52, 64, 65).

Microvascular endothelial cells, similar to tumor cells, are exposed to hypoxic and acidic environments (31, 60), which are not favorable for growth and cell survival. We have shown that pmV-ATPase expression in highly invasive metastatic tumor cells provides a dynamic $p_{\text{Hcyt}}$ regulatory mechanism for these cells (27, 51). The similarity between metastatic cells and angiogenic microvascular endothelial cells with regard to invasion of adjacent tissue by the invading cell led us to hypothesize that 1) microvascular, but not macrovascular, endothelial cells express pmV-ATPase as a dynamic $p_{\text{Hcyt}}$ regulatory mechanism, which allows them to cope with acidic environments, 2) microvascular endothelial cells employ this pump’s activity for cell migration, and 3) the presence of pmV-ATPases at the leading edge in microvascular endothelial cells allows them to maintain a more alkaline $p_{\text{Hcyt}}$ at the leading than at the lagging edge, thus creating a $p_{\text{Hcyt}}$ gradient favorable for cell migration.

**MATERIALS AND METHODS**

Medias, buffers, and chemicals. Dulbecco’s modified Eagle’s medium (DMEM) was supplemented with 10% or 20% fetal bovine serum (FBS), 2 mM L-glutamine, 5 mM d-glucose, 20 U/ml heparin, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.2 µg/ml amphotericin B (GIBCO, Grand Island, NY). Joklik’s essential medium (essentially Ca$^{2+}$-free) was supplemented with 60 mM taurine, 20 mM creatine, and 5 mM HEPES. Cell perfusion buffer (CPB) consisted of (in mM) 110 NaCl, 1 MgSO$_4$, 5.4 KCl, 1.5 CaCl$_2$, 0.44 KH$_2$PO$_4$, 0.35 NaH$_2$PO$_4$, 5 glucose, 2 L-glutamine, and 25 HEPES at the indicated pH. Na$^{+}$-free CPB consisted of all CPB ingredients, except those containing Na$^+$ (110 mM N-methylglucamine was substituted for NaCl). CPB solutions containing HCO$_3^-$

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were continuously bubbled with 5% CO₂ at 37°C. The concentration of HCO₃⁻ in the buffer was determined as described elsewhere (17). High-K⁺ buffer contained (in mM) 146 KCl, 20 NaCl, 5 glucose, 2 glutamine, 10 HEPEs, 10 MES, and 10 bicine. The rationale for using these organic buffers was to allow for precise buffering across a wide pH range (5.5–8.0) (27).

Bafilomycin A₁ was obtained from Wako Chemicals (Richmond, VA), and SCH-28080 was a generous gift of Dr. A. Barnett (Schering-Bloomfield, New Jersey). The fluorescent dyes were obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical (St. Louis, MO), unless otherwise stated.

Isolation of micro- and macrovascular endothelial cells. Microvascular (cardiac) and macrovascular (aortic) endothelial cells were isolated from normal BioBreeding (BB) rats (Biomedical Research Models, Worcester, MA) with use of techniques previously described, with some modifications (63). Briefly, for isolation of microvascular endothelial cells, heart ventricles were minced with fine surgical scissors and digested with Liberase Blendzyme 2 [Roche, Indianapolis, IN; 0.2 mg/ml in HEPEs-buffered DMEM (H-DMEM)] at 37°C for 60 min using a shaker for continuous agitation of the digestion solution. For isolation of macrovascular endothelial cells, aortic segments (dissected free of any attached connective tissue) were cut longitudinally and subjected to the digestion procedure used for the minced ventricles. Undigested tissue fragments were removed by filtration through 100-μm nylon mesh cell strainers (BD Biosciences, Franklin Lakes, NJ). The resulting cell suspensions were pelleted by centrifugation, rinsed twice, and resuspended in H-DMEM. Biotinylated mouse anti-rat CD31 (PECAM) antibody (clone 3A12, Research Diagnostics, Flanders, NJ) was incubated with 10 μM Dynabeads M-280, Invitrogen, Carlsbad, CA) were added, and the suspension was incubated for 45 min at room temperature on a rotator. The cells were again pelleted by centrifugation, rinsed, and resuspended in 1 ml of H-DMEM. Streptavidin-coated magnetic beads (10 μl; Dynabeads M-280, Invitrogen, Carlsbad, CA) were added, and the suspension was incubated for 45 min at room temperature on a rotator. For selection of endothelial cells, the Eppendorf tube containing the cell suspension was placed in a magnetic stand (magnetic particle concentrator, Invitrogen), and magnetic beads attached to endothelial cells were pulled to the side of the tube (adjacent to the magnet). Nonendothelial cells that remained in suspension were removed by aspiration. Beads were resuspended in H-DMEM, and the procedure was repeated twice to ensure removal of all contaminating nonendothelial cells. Endothelial cells were resuspended in growth medium and plated on gelatin-coated tissue culture dishes for cell growth expansion. Micro- and macrovascular endothelial cells from three to four rats were pooled into one 60-mm gelatin-coated (1.5% gelatin in PBS) petri dish and cultured at 37°C under 10% CO₂ in DMEM with 20% FBS. Endothelial cell identity was confirmed as described elsewhere (63). Micro- and macrovascular endothelial cells were passaged by trypsinization and subsequently grown at extracellular pH (pHe₅₇.4 in DMEM supplemented with HCO₃⁻ and 10% FBS.

Measurement of pH₅₇ in cell populations. pH₅₇ was determined by the fluorescence of carboxyseminalaphthodiuor-1 (5-[and 6]-carboxy-SNAF-1) as described previously (27). Briefly, two coverslips containing cells at confluency were loaded with 7.5 μM SNAF-1 in its acetoxymethyl ester (AM) form and incubated at 37°C in 5% CO₂ for 45 min and then in buffer for 30 min to ensure complete ester hydrolysis/leakage of uncleaved dye. The coverslips were placed in a holder-perfusion device, and the fluorescence of SNAF-1 was monitored with a spectrofluorometer (model SLM-8100/DMX) equipped for sample perfusion at 37°C. SNAF-1 was excited at 534 nm, with emission at 584 and 644 nm. The ratio of fluorescence at 644 nm to fluorescence at 584 nm was used to monitor pH changes. Fluorescence data were converted to ASCII format for subsequent data analysis in SigmaPlot (version 8.0, Jandel Scientific, San Rafael, CA).

In situ calibration of SNAF-1. In situ calibration curves were generated as described previously (27). Briefly, cells attached to coverslips were perfused with high-K⁺ buffers at pHe₅, 5.5–8.0 (at 2.0-pH unit intervals). The buffers contained 2 μM valinomycin and 6.8 μM nigericin to collapse the pH gradient. The ratio of SNAF-1 fluorescence at 644 nm to SNAF-1 fluorescence at 584 nm at each pH₅₇ was fitted to the following equation:

\[ \text{pH} = pK_a + \log \left( \frac{R_{\text{obs}} - R_{\text{min}}}{R_{\text{max}} - R_{\text{obs}}} \right) \]  \hspace{1cm} (1)

where R₉ is the fluorescence ratio at any given pH, R₉ is the fluorescence ratio when the dye is fully protonated, and pKₐ is the fluorescence ratio when the dye is fully unprotonated, and pKₐ is the apparent acid dissociation constant. Equation 1 is solved iteratively using nonlinear least squares analysis and yields pKₐ, R₉ and R₉ values for SNAF-1 in these cells. From these in situ calibration curves, the following parameters were obtained for SNAF-1 in microvascular endothelial cells (n = 33): pKₐ = 7.76 (SD 0.076), R₉ = 0.55 (SD 0.004), and R₉ = 2.49 (SD 0.23). The in situ calibration parameters for SNAF-1 in macrovascular endothelial cells (n = 33) were as follows: pKₐ = 7.69 (SD 0.089), R₉ = 0.435 (SD 0.007), and R₉ = 2.94 (SD 0.28). These values were significantly different between cell types (P < 0.05). pH₅₇ values were obtained for each experiment by using Eq. 1 and their corresponding in situ calibration parameters with SigmaPlot.

Measurement of pHe in discrete cellular regions with use of spectral imaging microscopy. Spectral imaging microscopy allows measurements of ions in discrete subcellular regions of single cells with high temporal, spectral, and spatial resolution (29, 49). The spectral imaging microscope is based on a Spectra-Pro-150 spectrograph directly coupled to the side port of an inverted microscope (model IX70, Olympus). The spectrograph has 300 grooves per nanometer of grating, is blazed at 500 nm (Acton Research, Acton, MA), and is equipped with a high-dynamic-range frame transfer back-illuminated charge-coupled device (CCD) camera (model Spec10B, Princeton Instruments, Trenton, NJ) controlled by an ST133 controller (Princeton Instruments). The CCD camera has a 1,340 × 512 pixel imaging array (pixel size = 9 × 9 μm). The entrance of the slit spectrograph was set at 0.2 mm throughout the experiments, except for the zero-order spectra, where the slit was set at 2.0 mm. The spectrograph and the CCD camera settings were computer controlled using commercially available software (WINSPEC/32 version 2.5, 10.1, Roper Scientific, Trenton, NJ). The CCD camera temperature was maintained at −100°C for all the experiments. The full spectral output of the cell can be obtained within as little as 2 ms and with 0.4-nm spectral resolution. The spatial information was obtained by alignment of a single cell along the length of the entrance slit, so that spectra were acquired from unique subcellular locations (i.e., leading edge to lagging edge). Data were collected from 15 discrete regions of interest of the cell and binned to obtain a higher signal-to-noise ratio. The optical filters were as follows: 510-nm-narrow band-pass filter and 550-nm-long band-pass dichroic filter (Omega Optical, Brattleboro, VT).

Immunocytochemistry. Micro- and macrovascular endothelial cells were fixed with 4% paraformaldehyde for 15 min, washed with 25 mM glycine, and then permeabilized with 0.1% Triton X-100. The cells were sequentially incubated with primary antibody specific for the E subunit of V-H⁺-ATPase (46). Cells were washed extensively and then labeled with Alexa Fluor 568 secondary (anti-mouse IgG) antibody and Alexa Fluor 488-phalloidin, which binds to F-actin and helps delineate the cell edge (51). The cells were mounted in Vecta-Mount solution (Vector Laboratories, Burlingame, CA) and maintained at 4°C overnight. The cells were observed with a confocal laser scanning microscope (model LSM 510 META, Zeiss) with a ×63 objective (Plan-APCHROMAT, 1.4 NA, oil differential interference contrast). Simultaneously acquired images of Alexa Fluor 488-phalloidin (actin cytoskeleton, green) and Alexa Fluor 568 (V-ATPase, red) fluorescence were collected, and each section was analyzed on a pixel-by-pixel basis utilizing Physiology software (version 3.0, Zeiss) to assess colocalization of actin and V-ATPase.
Cell migration/invasion assay. Microvascular, but not macrovascular, endothelial cells are involved in new blood vessel formation, which requires these cells to invade and migrate through extracellular matrix (ECM) proteins (20, 30, 51). To determine whether microvascular endothelial cells are more migratory and invasive than macrovascular endothelial cells, cells grown at confluence in T-25 flasks in DMEM were loaded with 5 µM calcein-AM for 30 min. The cells were then trypsinized, washed, and counted. To evaluate the degree of cell invasion through various ECMs in vitro, HTS FluoroBlok (Becton Dickinson, Bridgeport, NJ) inserts were briefly soaked in Matrigel, seeded at densities of $5 \times 10^5$ cells/well, and incubated at 37°C in 5% CO$_2$ for 24 h. HTS FluoroBlok inserts contain a 3-µm polyethylene terephthalate membrane impregnated with dyes that absorb visible light at 490–700 nm. To evaluate cell migration, we used this approach, except the filters were not coated with Matrigel. This allows us to study the ability of the cell to deform to allow it to migrate through the filter pores. The inserts were subsequently visualized, and images of the bottom and top of the insert were obtained with a ×20 objective (UPlan Fl 0.5 Ph1, Olympus) and a confocal microscope (model 1024 MRC, Bio-Rad, Hercules, CA). Calcein was excited with the 488-nm line of a 50-mW krypton-argon laser, and emission was collected using the VHS filter (Bio-Rad) blocks, which contain a 515-nm emission filter. Experiments were done in triplicate, and five images were obtained per HTS FluoroBlok. The images were subsequently analyzed, and the cells were visually counted in defined areas. Percent invasion/migration was corrected for proliferation and calculated as follows

\[
\text{% invasion} = \frac{\text{total no. of invading cells (lower well sample)/mm}^2}{\text{total no. of cells seeded (upper well sample)/mm}^2} \times 100\quad (2)
\]

We also evaluated cell migration using the wounded monolayer model in micro- and macrovascular endothelial cells (48, 51), which allows us to study the ability of cells to migrate to close the wound. The cells were grown on 12-mm coverslips to confluency and subsequently wounded with a micromanipulator to induce a 300-µm gap (51). The cells were allowed to close the wound for up to 24 h in the absence or presence of bafilomycin A1 to inhibit V-ATPase. At selected times, the cells were fixed, permeabilized, and incubated with FITC-phalloidin. Images of wounded monolayers were then obtained with a ×20 objective and a Bio-Rad confocal microscope (excitation at 488, emission at 515 nm). Migration was assessed as wound distance at selected times from three randomly selected areas.

Assay of cell-doubling times. Because differences in cell migration/invasion assays may be due to differences in cell growth between micro- and macrovascular endothelial cells, we evaluated cell growth. Cells were plated onto 24-well plates (Falcon, Becton Dickinson) at an initial density of $2 \times 10^4$ cells/well in triplicates. After 12 h, the cells were fixed with 1% glutaraldehyde to obtain values at time 0. Thereafter, the cells were fixed at 24-h intervals for up to 120 h. At the end of the experiment, the cells were stained with 0.1% crystal violet for 20 min, destained with running water for 5 min, and air-dried. The absorbance at 590 nm is linearly related to the number of cells; thus cell number can be estimated to obtain the kinetics of cell growth (16, 51). The data were fitted to the following sigmoid (3-parameter) equation to obtain the cell-doubling times using SigmaPlot software

\[
y = \frac{a}{1 + e^{(x - x_0)/b}}\quad (3)
\]

From these experiments, we determined that the doubling time in microvascular endothelial cells ($n = 4$) was 27.44 h (SD 2.24), whereas the doubling time in macrovascular endothelial cells ($n = 4$) was 40.45 h (SD 6.99). Thus doubling times were significantly faster in microvascular than in macrovascular endothelial cells ($P < 0.05$).

Statistical analysis. Data were analyzed by nonparametric and parametric tests and ANOVA (SigmaStat 2.03, Jandel Scientific, Richmond, CA) as appropriate. Statistical significance was assigned at $P < 0.05$.

RESULTS

Migration/invasion is greater in microvascular than in macrovascular endothelial cells. The degree of migration/invasion of cells through an artificial basement membrane matrix is significantly higher in microvascular than in macrovascular endothelial cells (Fig. 1, A and B). These experiments allowed us to study the ability of the cells to degrade ECM proteins and undergo dynamic morphological changes that would enable them to traverse the filter’s pores. We also performed wounding monolayer experiments to quantify the extent of cell migration after a wound. A wounded monolayer labeled with FITC-phalloidin at time 0 and 18 h after wound closure is shown in Fig. 1, C and D. From such experiments, we determined the extent of wound closure (i.e., migration) as a function of wound distance. We observed significantly more complete wound closure at 24 h in microvascular than in macrovascular endothelial cells ($n = 3$; Fig. 1E). Treatment of wounded monolayers with 20 nM bafilomycin A1 resulted in a significant inhibition of wound closure in microvascular, but not macrovascular, endothelial cells (Fig. 1, E and F). These data indicate that V-ATPases are important in the migration of microvascular endothelial cells.

Immunocytochemistry reveals pmV-ATPases in microvascular endothelial cells. For study of V-ATPase distribution, wounded monolayers of micro- and macrovascular endothelial cells were fixed, permeabilized, and labeled with Alexa Fluor 488-phalloidin (Fig. 2, A and D), and the E subunit of V-H$^+$-ATPase was secondarily labeled with Alexa Fluor 568 (Fig. 2, B and E). Sectional images (xyz simultaneous series) were collected, and each section was analyzed on a pixel-by-pixel basis with use of Physiology software (version 3.0) to assess the distribution of V-H$^+$-ATPases. The merge image of actin labeling and V-ATPase reveals V-H$^+$-ATPase at the leading edge in microvascular endothelial cells and emphasizes the absence of V-ATPase at the leading edge in macrovascular endothelial cells (Fig. 2, C and F; long arrows). These experiments were performed in wounded monolayers (to elicit polarization of the cell), where the leading (migratory) edge is on one side and the lagging edge on the opposite side of the wound. This approach allows us to study the distribution of V-ATPases in an artificially polarized cell monolayer. In macrovascular endothelial cells, V-ATPases are inconspicuous at the leading edge in polarized cells, which exhibit clearly defined lamellipodia (Fig. 2E). The merge image of actin and V-ATPase distribution emphasizes the absence of V-ATPase at the leading edge in macrovascular endothelial cells (Fig. 2F). Consistent with the presence of V-H$^+$-ATPases in intracellular organelles, intracellular compartments show abundant proton pumps in micro- and macrovascular endothelial cells.

Steady-state pH$_{cyt}$ is more alkaline in microvascular than in macrovascular endothelial cells. The degree of migration/invasion of cells through an artificial basement membrane matrix is significantly higher in microvascular than in macrovascular endothelial cells (Fig. 1, A and B). These experiments allowed us to study the ability of the cells to degrade ECM proteins and undergo dynamic morphological changes that would enable them to traverse the filter’s pores. We also performed wounding monolayer experiments to quantify the extent of cell migration after a wound. A wounded monolayer labeled with FITC-phalloidin at time 0 and 18 h after wound closure is shown in Fig. 1, C and D. From such experiments, we determined the extent of wound closure (i.e., migration) as a function of wound distance. We observed significantly more complete wound closure at 24 h in microvascular than in macrovascular endothelial cells ($n = 3$; Fig. 1E). Treatment of wounded monolayers with 20 nM bafilomycin A1 resulted in a significant inhibition of wound closure in microvascular, but not macrovascular, endothelial cells (Fig. 1, E and F). These data indicate that V-ATPases are important in the migration of microvascular endothelial cells.
maintain steady-state pHcyt (17). We determined that, at pHex 7.15, pHcyt was similar between microvascular [pHcyt 7.171 (SD 0.053)] and macrovascular [pHcyt 7.188 (SD 0.006)] endothelial cells (n = 5). To evaluate the contribution of the HCO3⁻-based H⁺-transport systems to the regulation of pHcyt, we performed experiments in HCO3⁻-free medium. We determined that the steady-state pHcyt values in micro- and macrovascular endothelial cells (n = 5) were 7.156 (SD 0.018) and 7.052 (SD 0.017), respectively, at pHex 7.15. Thus, in the absence of HCO3⁻, pHcyt was significantly higher in microvascular than in macrovascular endothelial cells (P < 0.05). Because pHex may affect pHcyt regulation, we performed experiments in the presence and absence of HCO3⁻ in micro- and macrovascular endothelial cells at pHex 6.5, 7.0, 7.15, and 7.4. In microvascular endothelial cells, pHcyt was unaffected by HCO3⁻, pHcyt in macrovascular endothelial cells was ~0.15 pH unit higher in the presence than in the absence of HCO3⁻ throughout the pH curve (i.e., pHex 6.5–7.4). These data suggest that pHcyt regulation is accomplished via distinct mechanisms in micro- and macrovascular endothelial cells.

Acid-loading experiments allow identification of Na⁺- and HCO3⁻-independent pHcyt recovery. To study the differences in the mechanisms of pHcyt regulation between micro- and macrovascular endothelial cells, we selected conditions in which the two main pHcyt regulatory mechanisms should be dormant. Thus acid-loading experiments utilizing the NH4Cl prepulse technique (34a), whereas acute removal of NH4Cl reversed the condition and caused rapid acidification in micro- and macrovascular endothelial cells. In the absence of Na⁺ and HCO3⁻, macrovascular endothelial cells (n = 11) did not recover from acidification [d[PH]/dt = 0.001 (SD 0.005), proton flux (JH⁺) = 0.01 mM H⁺/min (SD 0.1)], but microvascular endothelial cells did recover from this acid load (Fig. 3). The H⁺ buffering capacity (βi) was significantly higher in microvascular than in macrovascular endothelial cells (n = 11): βi = 36.7 (SD 1.08) vs. 30.9 (SD 1.37) mM (P < 0.05). To determine whether pHcyt recovery in microvascular endothelial cells was mediated by V-H⁺-ATPases, we examined pHcyt recovery from acid loads in an Na⁺- and HCO3⁻-free buffer in the presence of bafilomycin to inhibit V-H⁺-ATPase and found a significant decrease in JH⁺ (Fig. 3B). P-type H⁺-ATPase inhibitors such as SCH-28080 had no effect on JH⁺ (Fig. 3B). To determine whether Na⁺/H⁺ exchange and HCO3⁻-dependent H⁺ transport contributed to pHcyt regulation in microvascular endothelial cells, we performed acid-loading experiments in the presence of Na⁺ and HCO3⁻ and found JH⁺ values similar to those observed in medium with Na⁺ and without HCO3⁻ (cf. Fig. 3B). In the presence of Na⁺ and HCO3⁻, macrovascular endothelial cells also recovered from an acid load [JH⁺ = 1.44 (SD 0.34) mM H⁺/min (n = 5)]. These JH⁺ values are similar to those observed in microvascular endothelial cells. Collectively, these data indicate ubiquitous Na⁺- and HCO3⁻-dependent pHcyt regulatory mechanisms in micro- and macrovascular endothelial cells. Importantly, microvascular, but not macrovascular, endothelial cells exhibited an additional Na⁺- and HCO3⁻-independent pHcyt regulatory system that improved their ability to cope with acid loads (cf. Fig. 3, A and B). To further demonstrate that neither Na⁺/H⁺ exchange nor HCO3⁻-based H⁺-transporting mechanisms were involved in the pHcyt recoveries, we performed experiments in the absence of Na⁺ and HCO3⁻ with 5-(N,N-hexamethylene)-amiloride and DIDS, blockers of Na⁺/H⁺ exchange and anion transport, respectively (Fig. 3B). Neither 5-(N,N-hexamethylene)-amiloride (not shown) nor DIDS significantly altered the kinetics of...
pH<sub>cyt</sub> recovery in an Na<sup>+</sup>- and HCO<sub>3</sub><sup>-</sup>-free buffer. These data indicate the presence of an Na<sup>+</sup>- and HCO<sub>3</sub><sup>-</sup>-independent pH<sub>cyt</sub> regulatory mechanism in microvascular endothelial cells that allows them to recover from acid loads; this mechanism is absent in macrovascular endothelial cells.

Na<sup>+</sup> removal elicits a transient decrease in pH<sub>cyt</sub> in microvascular endothelial cells. Cell types that exhibit the Na<sup>+</sup>/H<sup>+</sup> exchanger as a major pH<sub>cyt</sub> regulatory system respond to acute Na<sup>+</sup> removal (in the absence of HCO<sub>3</sub><sup>-</sup>) with a rapid or a slow decrease in pH<sub>cyt</sub> (68). In the absence of Na<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>, most cells do not recover from this acidification (27). This is the case for macrovascular endothelial cells, which respond to Na<sup>+</sup> removal with a slow acidification [ΔpH<sub>cyt</sub> = 0.28 pH unit (SD 0.03, n = 6), half-time of acidification = 163 s (SD 30, n = 6); Fig. 4A]. Interestingly, Na<sup>+</sup> removal in microvascular endothelial cells resulted in a rapid acidification [ΔpH<sub>cyt</sub> = 0.17 pH unit (SD 0.03; n = 11)] followed by a rapid recovery to baseline levels (Fig. 4A). This recovery occurred in an HCO<sub>3</sub><sup>-</sup>-free buffer and was unaffected by preincubation with DIDS (Fig. 4B). Importantly, the pH<sub>cyt</sub> recovery in an Na<sup>+</sup>- and HCO<sub>3</sub><sup>-</sup>-free buffer was decreased by bafilomycin A<sub>1</sub>, a V-H<sup>+</sup>-ATPase inhibitor (Fig. 4B). These data suggest that the V-H<sup>+</sup>-ATPase was responsible for the pH<sub>cyt</sub> recovery from an acid load in microvascular endothelial cells and that Na<sup>+</sup>/H<sup>+</sup> exchange is the likely pH<sub>cyt</sub> regulatory mechanism used by macrovascular endothelial cells.

Spectral imaging microscopy shows a more alkaline pH<sub>cyt</sub> at the leading than at the lagging edge of the cell in wounded monolayers. Because microvascular, but not macrovascular, endothelial cells exhibit pmV-ATPase as a unique pH<sub>cyt</sub> regulatory mechanism, we focused on microvascular endothelial cells to evaluate whether there is a distinct pH<sub>cyt</sub> regulation in domains exhibiting pmV-ATPase. These studies were performed in wounded monolayers (cf. Fig. 1C), because, after they are wounded, the cells move forward to close the wound, creating a polarized system where the leading edge is at the wound site and the lagging edge is at the rear of the cell. The immunocytochemical evidence of pmV-ATPase at the leading edge of the cell in wounded monolayer experiments prompted us to hypothesize that the localization of pmV-ATPase at the leading edge might result in a distinct pH<sub>cyt</sub> gradient from the leading to the lagging edge of the cell (cf. Fig. 2). To eliminate any bias in our interpretation of the data regarding differences in pH<sub>cyt</sub> (which could be due to differences in dye concentration and/or intracellular environment that may be distinct at the leading or lagging edge of the cell), we performed spectral imaging experiments in SNARF-1-loaded wounded monolayers. This approach allows us to monitor the full spectral output of the pH indicator from the wounded (leading) to the rear (lagging) edge of the cell. The spectral properties of SNARF-1 (i.e., relative distance of the ion-sensitive spectral shoulders at 584 and 644 nm) are only sensitive to H<sup>+</sup> concentration and unaffected by dye concentration (19). Figure 5A shows a low-magnification area of the wounded monolayer aligned along the slit entrance of the spectrograph. Decreasing the slit width from 2,000 μm (Fig. 5B) to 1,000 μm (Fig. 5C), and then to 200 μm (Fig. 5D), provides spatial information from the leading to the lagging edge of the cell (cf. Fig. 5, A–C). Figure 5E shows the first-order spectra of Fig. 5D. For these experiments, we binned 15 regions of interest, each corre-
Microvascular, but not macrovascular, endothelial cells exhibit pmV-ATPases for pHcyt regulation, as demonstrated by pharmacological and ion-substitution experiments. Thus pmV-ATPase is a novel mechanism that allows microvascular endo-

sponding to ~5 μm across the length of the cell from the leading to the lagging edge. For purposes of data presentation, only two spectra, corresponding to the leading and lagging edges, are shown (Fig. 5F). From in situ calibrations performed at the end of the experiment, we concluded that, under steady-state conditions, the spectral shape of SNARF-1 was more alkaline (by ~0.2 pH unit) in the leading than in the lagging edge (cf. Fig. 5F). The salient spectral properties of SNARF-1 show the predicted behavior for this ratiometric dye, i.e., increases and decreases in the fluorescence signal at 644 and 584 nm, respectively, as pH is increased. The more alkaline pH gradient at the leading than at the lagging edge of the cell is sustained (Fig. 5G). NH₄Cl elicited a cytosolic alkalinization, and its removal induced a cytosolic acidification in the absence of Na⁺ and HCO₃⁻. The magnitude of the pHcyt changes after NH₄Cl treatment and its removal was larger in the lagging than in the leading edge of the cell, consistent with lower H⁺ buffering capacity in the lagging edge of the cell.

The validity of these estimations on pHcyt relies on the ability to fully collapse the pHcyt gradients across all compartments. We have performed complete in situ titrations at discrete distances of the cell from the leading to the lagging edge (i.e., at ~5-μm intervals) and have utilized in situ calibration parameters for each of these regions. This type of calibration is needed, because fluorescent ion indicators have been reported to exhibit distinct pKₐ in distinct cell types (16, 27). Thus this approach should minimize errors inherent to distinct dye concentration and intracellular environment (e.g., viscosity and protein binding) that may exist in discrete cellular regions from the leading to the lagging edge. From a number of in situ titrations similar to those shown in Fig. 6A, we determined that there are no significant differences in pKₐ of the dye in any of the regions studied, indicating that the pHcyt gradients were fully collapsed (Fig. 6B). There are, however, significant differences in Rmax and Rmin (Fig. 6C). Together, the data indicate that the distinct pHcyt values observed at the leading and lagging edges are due to distinct pHcyt regulation in these regions.

DISCUSSION

Dynamic vascular remodeling during angiogenesis requires growth and invasion of endothelial cells into tissues. However, the acidic extracellular environment that prevails in angiogenesis is not conducive for growth. This study demonstrates that microvascular, but not macrovascular, endothelial cells employ pmV-ATPases for pHcyt regulation, as demonstrated by pharmacological and ion-substitution experiments. Thus pmV-ATPase is a novel mechanism that allows microvascular en-

![Fig. 3](http://ajpheart.physiology.org/)

**Fig. 3.** A: microvascular, but not macrovascular, endothelial cells exhibit a Na⁺- and HCO₃⁻-independent cytosolic pH (pHcyt) regulatory system. Cells were grown on glass coverslips to confluency, intracellularly loaded with carboxysemaphorin-difluor-1 (SNARF-1)-AM, and transferred to a spectrophotometer for pHcyt measurements. Cells were superfused with cell perfusion buffer (CPB) until steady-state pHcyt was reached. At 1st arrow, superfusate was exchanged with 25 mM NH₄Cl. At 2nd arrow, superfusate was exchanged for Na⁺- and HCO₃⁻-free CPB. Data are representative of 34 and 11 experiments on micro- and macrovascular endothelial cells, respectively. B: effect of inhibitors of primary and secondary H⁺ transport systems on proton flux (JpH) in microvascular endothelial cells. Cells were handled as described in A. At the time indicated by 2nd arrow in A, superfusate was exchanged with Na⁺- and HCO₃⁻-free CPB containing 50 μM bafilomycin (n = 6), 100 μM DIDS (n = 3), or 5 μM SCH-28080 (n = 5). Recovery of pHcyt (JpH) after acid loading were determined during the first 3 min from experiments similar to those described in A. JpH was obtained by multiplying dpH/dt in the first 5 min of recovery by apparent intrinsic buffering capacity (β) as described elsewhere (17). Values are means ± SE. *P < 0.05 vs. Na⁺- and HCO₃⁻-free medium.

![Fig. 4](http://ajpheart.physiology.org/)

**Fig. 4.** A: effect of acute Na⁺ removal in micro- and macrovascular endothelial cells. Cells were handled as described in Fig. 3A, except at the arrow, superfusate was changed to Na⁺-free CPB. Data are representative of 11 and 6 experiments for micro- and macrovascular endothelial cells, respectively. Microvascular, but not macrovascular, endothelial cells recover from Na⁺-induced acidification. B: effect of inhibitors on JpH in microvascular endothelial cells. Recovery of pHcyt after removal of Na⁺ (JpH) was estimated during the first 3 min from experiments described in A. Values are means ± SE of 3 experiments for 100 μM DIDS and 5 experiments for 50 nM bafilomycin. *P < 0.05 compared with Na⁺-free medium.
dothelial cells to cope with the acidic extracellular environment. Recently, Otani et al. (38) showed that V-H\(^+\) H\(_{11001}\)-ATPases play a crucial role in growth and phenotypic modulation of myofibroblasts that contribute to neointimal formation in cultured human saphenous vein. This process also requires extensive vascular remodeling and involves several cell types, including myofibroblasts, fibroblasts, smooth muscle cells, and endothelial cells (38). Otani et al. also showed that the 16-kDa subunit of the V-H\(^+\) H\(_{11001}\)-ATPase was overexpressed predominantly in the perinuclear region of the myofibroblasts. The 16-kDa subunit is considered to be the principal component of the V\(_0\) membrane channel sector of the V-H\(^+\) H\(_{11001}\)-ATPase, which is located predominantly in acidic organelles, lysosomes, and the trans-Golgi network (36). The main function of the V-H\(^+\) H\(_{11001}\)-ATPase in these organelles is maintenance of an acidic pH, which is important for several physiological processes, including endocytosis, exocytosis, intracellular trafficking, growth, and differentiation (32, 36). Thus our study extended these observations to indicate that pmV-ATPase plays a role in regulating pH\(_{cyt}\), in addition to its well-recognized role in regulating the acidity of intracellular organelles.

Because migration and invasion through the ECM are important elements in angiogenesis, we evaluated whether microvascular endothelial cells could penetrate an artificial basement membrane more readily than macrovascular endothelial cells. Our data indicated that microvascular endothelial cells exhibiting pmV-ATPase were more migratory/invasive than macrovascular endothelial cells, which do not exhibit pmV-ATPase. Because vascular remodeling after a lesion involves migration of endothelial cells from the noninjured area to repair the lesion, we employed a wounded monolayer experiment. In this experiment, scraping off a 300-μm region in a confluent monolayer of endothelial cells results in cell migration toward the damaged region to repair the wound (48). This orderly movement of cells adjacent to the lesion occurs immediately and reveals an easily distinguishable leading edge. When
healing was allowed to continue, the wound was closed in ∼24 h. Importantly, bafilomycin treatment significantly decreased wound closure in microvascular endothelial cells. These data indicate that a bafilomycin-sensitive component is involved in migration of microvascular endothelial cells.

Because a faster rate of wound closure could be explained by distinct doubling times between micro- and macrovascular endothelial cells, we evaluated this parameter. Our data indicated that doubling time in microvascular endothelial cells is ∼27 h, whereas doubling time in macrovascular endothelial cells is ∼40 h. Wound closure in 24 h in microvascular endothelial cells and doubling times >24 h in micro- and macrovascular endothelial cells support our contention that the different rates of wound closure are not due to differences in cell growth between micro- and macrovascular endothelial cells but, rather, to a distinct difference in migratory rate between these cell types.

The data reported by Bai et al. (3) suggest that wound closure is faster in microvascular than in macrovascular endothelial cells. Bai et al. estimated that human microvascular endothelial cells migrate at ∼15 μm/h, whereas human umbilical vein endothelial cells (HUVEC) migrate at ∼10 μm/h under nonstimulating conditions. There are, however, controversies regarding rates of cell migration. Specifically, Morales et al. (34) reported 27% wound closure within 24 h in wounded monolayers of HUVEC. In this case, the wound size was ∼2.5 mm. However, faster migration rates in a wounded monolayer of HUVEC and human coronary aortic endothelial cells, where wound closure is ∼40% and 30%, respectively, within 6 h have also been reported (1). The wound size in these other studies was ∼150–200 μm. Thus it appears that a major reason for the different rate of migration is wound size, because larger wounds are associated with slower migration rates, possibly because of the release of chemoattractants from cells that work in a paracrine fashion stimulating cell migration. It is possible that distinct differences in rates of wound closure between micro- and macrovascular endothelial cells may be due to distinct sensitivities of microvascular endothelial cells to chemo- kines (59). Microvascular endothelial cells from lung and kidney produce more chemokines, such as fractalkine, interleukin-1, tumor necrosis factor-α, and interferon-γ, than macrovascular endothelial cells (HUVEC) (5). Human dermal microvascular endothelial cells are more sensitive to cytokines, such as oncostatin and IL-6, basic fibroblast growth factor, and IL-1β, than HUVEC (59). Significant cell division as a mechanism of wound healing is not likely to occur in cells before 15–24 h (1, 25), because endothelial cells exhibit slower doubling times. Thus our study extends previous observations by indicating that expression of pmV-ATPase at the leading edge in microvascular endothelial cells is a mechanism that explains the faster rates of migration in microvascular than in macrovascular endothelial cells.

Our observations that bafilomycin decreases the rate of cell migration are in agreement with a recent study in which higher concentrations of bafilomycin (∼100–500 nM) than those used in our study suppressed cell motility in NIH 3T3 A31 mouse fibroblasts (57). The authors hypothesized that the effect of bafilomycin on cell motility was due to alterations of pH gradients in endocytic structures, which are known to exhibit V-ATPase. Recently, endosome fusion to the plasma membrane has been suggested as an important mechanism for wound healing in fibroblasts (45). Furthermore, overexpression of the 16-kDa subunit of V-ATPase in 10T1/2 fibroblasts has been shown to enhance invasion and the secretion of matrix metalloproteinase-2, an enzyme needed for protein degradation during invasion (23). Although the subcellular location of the overexpressed 16-kDa subunit was not evaluated in that study, these data indicate that overexpression of V-ATPase may be important for invasion. Our immunocytochemical data show that V-ATPase colocalizes with actin filaments at the cell’s cortex and at the leading edge. This is consistent with previous studies that have indicated that V-ATPase colocalizes with actin at the cell’s cortex in the ruffled border of activated osteoclasts (21, 24) and in the apical region of the middle gut epithelium of Manduca (62). In human breast cancer cells, we recently showed that pmV-ATPase expression is important for migration/invasion of highly metastatic human breast cancer cells (51). Therefore, our study complements these observations to indicate that pmV-ATPase is important for migration in microvascular endothelial cells.

Regulation of pH_{cyt} in most cells is accomplished by the relative contribution of Na⁺/H⁺ exchanger and HCO₃⁻-based H⁺-transporting mechanisms (18, 44, 47). Microvascular endothelial cells are not the exception, because they exhibited Na⁺/H⁺ exchanger and HCO₃⁻-based H⁺-transporting mechanisms. In addition to these important pH_{cyt}, regulatory mechanisms, pmV-ATPases are also used to regulate pH_{cyt} in microvascular, but not macrovascular, endothelial cells. Furthermore, use of wounded monolayer experiments to reveal the leading edge of migrating cells indicate that pmV-ATPase is present at the leading edge. As a result, cells exhibit a more alkaline pH_{cyt} at the leading than at the lagging edge. Differences in pH_{cyt} regulation at the leading and lagging edges are predicted by flux ratio equations, because the passive H⁺ influx is ∼45 and 56 times the passive efflux at the leading edge and lagging edges, respectively (if it is assumed that membrane potential is ∼90 mV and pH_{cyt} values in Fig. 5G are used for leading and lagging edges at pH_{e}, 7.4). This suggests that H⁺ influx is larger at the lagging edge, consistent with a more dynamic pH_{cyt} regulatory system at the leading edge. Further support for a dynamic mechanism to maintain such pH_{cyt} differences in leading and lagging edges is based on the fact that although H⁺ permeability is extremely high (10⁻³ cm/s), the actual J_{H⁺} across the plasma membrane is very low because of the low free H⁺ concentration in the cytosol and in the extracellular environment (if we assume pH_{e}, 7.4). Under these conditions, the passive H⁺ influx is ∼0.02 pH unit/h, yet the observed difference in pH_{cyt} between the leading and the lagging edge is ∼0.2 pH unit within the time frame of our experiments (i.e., ∼5–20 ms). Thus it is unlikely that such differences in pH_{cyt} values from the leading to the lagging edge are due to simple H⁺ diffusion. We interpret these data to suggest that pmV-ATPase at the leading edge is a dominant pH_{cyt} regulatory system that allows these pH_{cyt} gradients to exist in microvascular endothelial cells.

The variation in the steady-state deprotonated-to-protonated SNARF-1 ratio may be due to actual pH_{cyt} differences, variations in regional cytoplasmic microviscosity (28, 43, 55, 66), or even a different proportion of dye bound to cytoplasmic proteins (4). To properly interpret the differences in SNARF-1 protonated-to-deprotonated ratios, we have taken into account...
the behavior of the pH fluor probe in the cytoplasm, because it is heterogeneous in terms of composition and organization. Regional intracellular microenvironments may differ in viscosity, which in turn could result in distinct behavior of the fluor probes (39). Indeed, it is known that ion-sensitive fluor probes may display spectral differences not only between in vitro and in situ environments (7, 42), but also within the distinctive intracellular organelles (2, 12, 40, 56). Viscosity values for the leading edge (3.8 mPa-s), lagging edge (0.5 mPa-s), and soma (0.5 mPa-s) of locomoting neutrophils have been documented (66). Thus regional differences in cytoplasmic viscosity and/or the interactions between cytoplasmic proteins and the fluorescent dye may contribute to regional variations in the ratio and the in situ calibration parameters (pK′, Rmax, and Rmin) used to estimate pHcyt.

Important effects of protein on the in vitro calibration parameters have been described for several ion indicators, including pH fluor probes (4, 41, 54). However, it has also been suggested that SNARF-1 does not bind to bovine serum albumin but, rather, that a contaminant present in the commercially available SNARF-1 binds to bovine serum albumin (67). Furthermore, in the cytoplasm of cardiac myocytes, a major fraction of the fluor probes (0.5–0.9) appears to be bound to albumin but, rather, that a contaminant present in the commercial availability SNARF-1 binds to bovine serum albumin (67).

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H1156  
MICROVASCULAR ENDOTHELIAL CELLS EXHIBIT pmV-ATPASES


25.  H1156 MICROVASCULAR ENDOTHELIAL CELLS EXHIBIT pmV-ATPASES


