Vacuolar Type H\textsuperscript{+}-ATPases at the Plasma Membrane Regulate pH and Cell Migration in Microvascular Endothelial Cells

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ABSTRACT

Microvascular endothelial cells involved in angiogenesis are exposed to an acidic environment that is not conducive for growth and survival. These cells must exhibit a dynamic intracellular pH (pH\textsuperscript{cyt}) regulatory mechanism to cope with acidosis, in addition to the ubiquitous Na\textsuperscript{+}/H\textsuperscript{+} exchanger and HCO\textsubscript{3}⁻-based H\textsuperscript{+}-transporting systems. We hypothesize that microvascular endothelial cells exhibit plasmalemmal vacuolar-type proton ATPases (pmV-ATPases) to better cope with this acidic environment and that pmV-ATPases are required for cell migration. This study indicates that microvascular endothelial cells that are more migratory than macrovascular endothelial cells express pmV-ATPases. Spectral imaging microscopy indicates that the leading edge of microvascular endothelial cells exhibits a more alkaline pH\textsuperscript{cyt} than the lagging edge. Treatment of microvascular endothelial cells with V-ATPase inhibitors decreases both the proton fluxes via pmV-ATPases and cell migration. These data suggest that pmV-ATPases are essential for pH\textsuperscript{cyt} regulation and cell migration in microvascular endothelial cells.
INTRODUCTION

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 needed for the formation of new capillary networks, a necessary step in vascular remodeling (13).

Changes in intracellular pH ($pH_{cyt}$) are important in signal transduction mechanisms that 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 $pH_{cyt}$ 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). Some specialized and highly invasive cells (metastatic cells, macrophages, neutrophils, and osteoclasts) also utilize plasma membrane V-H$^+$-ATPases (pmV-ATPases) to regulate $pH_{cyt}$ (27, 35, 50, 58). These ATPases are distinguished from other proton pumps by their pharmacologic inhibition (8, 34). The V-type ATPases are inhibited by bafilomycin A$_1$, concanamycin, salicylihalamide, and 7-chloro-4-dinitrobenz-2-oxa-1,3-diazole [NBD-Cl] which have no effect on the P- or F-type ATPases (6, 9, 52, 64, 65).

Microvascular endothelial cells, like 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 $pH_{cyt}$ 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: (a) micro- but not macrovascular endothelial cells express pmV-ATPase as a dynamic pH regulatory mechanism that allows them to cope with acidic environments; (b) microvascular endothelial cells employ this pump’s activity for cell migration; and (c) the presence of pmV-ATPases at the leading edge in microvascular endothelial cells allows them to maintain a more alkaline pH in the leading than in the lagging edge, thus creating a pH gradient favorable for cell migration.
MATERIALS AND METHODS

Media, 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 Ca²⁺-free) was supplemented with 60 mM taurine, 20 mM creatine, and 5 mM HEPES. Cell Perfusion Buffer (CPB) consisted of 110 mM NaCl, 1 mM MgSO₄, 5.4 mM KCl, 1.5 mM CaCl₂, 0.44 mM KH₂PO₄, 0.35 mM NaH₂PO₄, 5 mM glucose, 2 mM L-glutamine, and 25 mM HEPES, at the indicated pH. Na⁺-free CPB consisted of all CPB ingredients, except those containing sodium. N-methyl-glucamine (110 mM) was used to substitute for NaCl. CPB solutions containing HCO₃⁻ were continuously bubbled with 5% CO₂ at 37°C. The concentration of HCO₃⁻ in the buffer was determined as described earlier (17). High K⁺ buffer contained 146 mM KCl, 20 mM NaCl, 5 mM glucose, 2 mM glutamine, 10 mM HEPES, 10 mM MES, and 10 mM Bicine. The rationale for using these organic buffers was to allow for precise buffering across a wide pH ranging from 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, NJ). 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 Macro-vascular Endothelial Cells.

Both microvascular (cardiac) and macrovascular (aortic) endothelial cells were isolated
from normal BioBreeding (BB) rats (Biomedical Research Models, Inc., Worcester MA) using
techniques previously described, with some modifications (63). Briefly, to isolate 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 Dulbecco’s
modified Eagle’s medium (H-DMEM)] at 37°C for 60 minutes using a shaker for continuous
agitation of the digestion solution. To isolate macrovascular endothelial cells, aortic segments
(dissected free of any attached connective tissue) were cut longitudinally and put through the
same digestion procedure as the minced ventricles. Undigested tissue fragments were removed by
filtering through 100 µm nylon mesh cell strainers (BD Biosciences, Bedford, MA). The resulting
cell suspension was pelleted by centrifugation, rinsed twice, and resuspended in H-DMEM.
Biotinylated mouse anti-rat CD31 (PECAM) antibody (clone 3A12, Research Diagnostics,
Flanders, NJ) was added to a concentration of 5 µg/ml and the suspension was incubated for 60
min at room temperature on a rotator. The cells were again pelleted by centrifugation, rinsed and
resuspended in 1 ml 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 the rotator. Endothelial cells were selected by placing the eppendorf tube
containing the cell suspension in a magnetic stand (magnetic particle concentrator, Invitrogen).
Magnetic beads attached to endothelial cells were pulled to the side of the tube (adjacent to the
magnet). Non-endothelial cells that remain in suspension were removed by aspiration. Beads were
resuspended in H-DMEM and the procedure was repeated twice to ensure that all contaminating
non-endothelial cells have been removed. Endothelial cells were resuspended in growth medium
and plated on gelatin-coated tissue culture dishes for cell growth expansion. Microvascular 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 were cultured at 37°C under 10% CO₂ in DMEM with 20% FBS. Endothelial cell identity was confirmed as described (63). Microvascular and macrovascular endothelial cells were passaged by trypsinization and subsequently grown at pH_{ex} 7.4 in DMEM supplemented with HCO₃⁻ and 10% FBS.

**pH_{ex} Measurements in Cell Populations.**

pH_{ex} was determined by the fluorescence of SNARF-1 (5-[and-6] carboxy-SNARF-1) as described previously (27). Briefly, two cover slips containing cells at confluency were loaded with 7.5 µM SNARF-1 in its acetoxymethyl (AM) ester form and incubated at 37°C in 5% CO₂ for 45 minutes followed by further incubation in buffer for 30 minutes to ensure complete ester hydrolysis/leakage of uncleaved dye. The cover slips were placed in a holder/perfusion device and the fluorescence of SNARF-1 was monitored with a SLM-8100/DMX spectrofluorometer equipped for sample perfusion, at 37°C. SNARF-1 was excited at 534 nm and the emissions at 584 and 644 nm. The ratio of 644/584 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 SNARF-1.

*In situ* calibration curves were generated as described previously (27). Briefly, cells attached to cover slips were perfused with high K⁺ buffers ranging in pH_{ex} from 5.5 to 8.0 (at ca. 0.2 pH unit intervals). The buffers contained 2 µM valinomycin and 6.8 µM nigericin, to collapse
the pH gradient. The ratio (644/584) values of SNARF-1 at each pH value were fitted into the following equation:

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

where $R_{\text{obs}}$ is the ratio observed at any given pH, $R_{\text{min}}$ is the ratio observed when the dye is fully protonated, $R_{\text{max}}$ represents the ratio of fluorescence obtained when the dye is fully unprotonated, and $pK_a'$ represents the apparent acid dissociation constant. The equation is solved iteratively using nonlinear least squares analysis, and yields the values of $pK_a'$, $R_{\text{min}}$, and $R_{\text{max}}$ for SNARF-1 in these cells. From these in situ calibration curves, the following parameters were obtained for SNARF-1 in microvascular endothelial cells: $pK_a' = 7.76$ (SD 0.076; $n = 33$), $R_{\text{min}} = 0.55$ (SD 0.004; $n = 33$), and $R_{\text{max}} = 2.49$ (SD 0.23; $n = 33$). The in situ calibration parameters for SNARF-1 in macrovascular endothelial cells were as follows: $pK_a' = 7.69$ (SD 0.089; $n = 33$), $R_{\text{min}} = 0.435$ (SD 0.007; $n = 33$), and $R_{\text{max}} = 2.94$ (SD 0.28; $n = 33$). These values were significantly different between cell types ($P < 0.05$). Intracellular pH values were obtained for each experiment by using equation [1] and their corresponding in situ calibration parameters with SigmaPlot.

**pH**\textsuperscript{cyt} Measurements in Discrete Cellular Regions Using Spectral Imaging Microscopy.

This approach 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 the following: a Spectra-Pro-150 spectrograph directly coupled to the side port of an Olympus IX70 inverted microscope. The spectrograph has 300 groves per nm grating and is blazed at 500 nm (Acton Research, Acton MA, equipped with a high dynamic range frame transfer back illuminated Charge Coupled Device (CCD) camera (Spec10B; Princeton)
Instruments, Trenton, NJ) which is controlled by an ST133 controller (Princeton Instruments). The CCD has a 1340 x 512 pixel imaging array (pixel 9 x 9 μm). The entrance of the slit spectrograph is set at 0.2 mm throughout the experiments, except for the zero order spectra, where the slit is set at 2.0 mm. The spectrograph and the CCD camera settings are computer controlled using commercially available software (Winspec/32 ver 2.5.10.1, Roper Scientific, Trenton NJ). The CCD temperature is maintained at -100 °C for all the experiments. The full spectral output of the cell can be obtained within a time frame of as little as 2 msec and with 0.4 nm spectral resolution. The spatial information is obtained by aligning a single cell along the length of the entrance slit, so that spectra is acquired from unique subcellular locations (i.e. leading to lagging edge). Data were collected from 15 discrete regions of interest (ROI) of the cell and binned to obtain a higher signal/noise ratio. The optical filters were as follows: 510 nm narrow bandpass filter; 550 long bandpass dichroic (Omega Optical, Brattleboro, VT).

**Immunocytochemistry.**

Macrovascular and microvascular 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) and Alexa fluor 488--phalloidin that binds to F actin and helps to delineate the cell edge (51). Cells were mounted in VectaMount solution (Vector laboratories, Burlingame, CA) and maintained at 4°C overnight. The cells were observed with a confocal laser scanning microscope (LSM 510 META, Zeiss) with a 63x objective (Plan-APOCHROMAT, 1.4 N.A., oil
DIC). 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 V 3.0 (Zeiss) to assess co-localization of actin and V-ATPase.

**Cell Migration/Invasion Assay.**

Microvascular but not macrovascular endothelial cells are involved in new blood vessel formation that requires the ability of these cells to invade and migrate through extracellular matrix (ECM) proteins (20, 30, 51). To determine if 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 minutes. 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 × 10^4, and incubated at 37°C / 5% CO₂ for 22 hours. HTS FluoroBlok™ inserts contain a 10 μm polyethylene terephthalate (PET) membrane impregnated with dyes that absorb visible light from 490-700 nm. To evaluate cell migration, we used this approach except that filters were not coated with matrigel. This allows to study the ability of the cell to undergo cell-deformability to migrate through the filter pores. The inserts were subsequently visualized and images of the bottom and top of the insert obtained with a 20x/0.5 Ph1 objective (Olympus Uplan Fl) and a Bio-Rad 1024 MRC confocal microscope (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 (BioRad) blocks which contain a OG515 emission filter. Experiments were done in
triplicate and five images were obtained per HTS FluoroBlok™. The images were subsequently analyzed and cells visually counted in defined areas. Percent invasion/migration was corrected for proliferation and calculated by using the following equation:

\[
\%\text{ Invasion} = \frac{\text{Total \# invading cells (lower well sample)} / \text{mm}^2}{\text{Total \# of cells seeded (upper well sample)} / \text{mm}^2} \times 100
\]

We also evaluated cell migration using the wounded monolayer model in microvascular and macrovascular endothelial cells (48, 51). This allows to study the ability of cells to migrate to close the wound. Cells were grown on 12 mm cover slips to confluence and subsequently wounded using a micromanipulator to induce a 300 μm gap (51). Cells were allowed to close the wound for up to 24 hours in the absence or presence of bafilomycin, to inhibit V-ATPase. At selected time points, cells were fixed, permeabilized, and incubated with FITC-phalloidin. Images of wounded monolayers were then obtained with a 20x objective and a Bio-Rad confocal microscope (ex 488; em OG515). Migration was assessed as wound distance at selected time points from 3 randomly selected areas.

**Cell Doubling Times Assay.**

Differences in cell migration/invasion may be due to differences in cell growth between microvascular and macrovascular endothelial cells. We therefore evaluated cell growth in these cells. Cells were plated onto 24 well plates (Falcon, Becton Dickinson, Bridgeport, NJ) at an initial density of 2 x 10^4/well in triplicates. Twelve hours thereafter, cells were fixed with 1%
glutaraldehyde to obtain time = 0. Thereafter, cells were fixed at 24hrs intervals for up to 120 hrs. At the end of the experiment, cells were stained with 0.1% crystal violet for 20 min, de-stained 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 parameters) equation to obtain the cell doubling times using Sigmaplot software:

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

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

Statistical Analysis.

Data were analyzed by non-parametric and parametric tests and ANOVA, as needed (SigmaStat 2.03; Jandel Scientific, Richmond, CA). 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 1A, 1B**). These experiments allowed to study the ability of cells to degrade extracellular matrix proteins and to undergo dynamic morphological changes to traverse the filter’s pores. We also performed wounding monolayer experiments to quantify the extent of cell migration following a wound. **Figures 1C and 1D** show a wounded monolayer labeled with FITC-phalloidin at t = 0 (**Fig 1C**) and after 18 hours (**Fig 1D**) of wound closure, respectively. From this type of experiments, we determined the extent of wound closure (i.e., migration) as a function of wound distance. We observed that the wound closure at 24 hrs in microvascular endothelial cells is significantly faster than in macrovascular endothelial cells (n = 3; **Fig 1E**). Treatment of wounded monolayers with 20 nM bafilomycin A_1_ resulted in a significant inhibition of wound closure in microvascular, but not in macrovascular endothelial cells (**Fig 1E, 1F**). These data indicate that V-ATPases are important in the migration of microvascular endothelial cells.

**Immunocytochemistry reveals pmV-ATPases in microvascular endothelial cells.** To study the distribution of V-ATPase, wounded monolayers of micro- and macro-vascular endothelial cells were fixed, permeabilized and labeled with Alexa-phalloidin (**Fig 2A, 2D**) and E subunit of V-H^+^-ATPase secondarily labeled with Alexa-fluor 568 (**Fig 2B, 2E**). Sectional images (XYZ simultaneous series) were collected and each section was analyzed on a pixel by pixel basis utilizing Physiology software V 3.0 (Zeiss), to assess the distribution of V-H^+^-ATPases. The
merge image of actin-labeling and V-ATPase revealed V-H\(^+\)-ATPase at the leading edge in microvascular endothelial cells and emphasize the absence of V-ATPase at the leading edge of macrovascular endothelial cells (Fig 2C, 2F; long arrows). These experiments were performed in wounded monolayers (to elicit polarization of the cell), where the leading and migratory edge is at the side of the wound and the lagging edge is on the opposite site of the wound. Notice a clearly defined lamellipodia at the wound site (long arrows). This approach allows us to study the distribution of V-ATPases in an artificially polarized monolayer of cells. Notice that the pmV-ATPases are conspicuous at the leading edge of microvascular endothelial cells (long white arrows), whereas the V-ATPases are incospicuous at the lagging edge of the same cell (short arrows) (Fig 2B, 2C). The cells behind the wound do not show apparent V-ATPase at their plasma membranes (short arrows). In macrovascular endothelial cells, V-ATPases are incospicuous at the leading edge in polarized cells that 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 both microvascular and macrovascular endothelial cells.

**Steady-state pH\(^{st}\) is more alkaline in microvascular than in macrovascular endothelial cells in the absence of HCO\(_3^-\).** The pH\(^{st}\) regulation of endothelial cells is thought to be mediated by the Na\(^+\)/H\(^+\)-exchanger and HCO\(_3^-\) -based H\(^+\) transport systems. To evaluate pH\(^{st}\) regulation, endothelial cells were perfused with media containing Na\(^+\) and HCO\(_3^-\). Under these conditions all pH\(^{st}\) regulatory mechanisms operate to maintain steady-state pH\(^{st}\) (17). We
determined that at a pH\(^{\text{ex}}\) = 7.15, the pH\(^{\text{cyt}}\) was similar between microvascular [pH\(^{\text{cyt}}\) = 7.171 (SD 0.053; n = 5)] and macrovascular [pH\(^{\text{cyt}}\) = 7.188 (SD 0.006; n = 5)] endothelial cells. To evaluate the contribution of the HCO\(_3^-\)-based H\(^+\)-transport systems in the regulation of pH\(^{\text{cyt}}\), we performed experiments in a HCO\(_3^\)-free media. We determine that the steady-state pH\(^{\text{cyt}}\) values in microvascular and macrovascular endothelial cells were 7.156 (SD 0.018; n=5) and 7.052 (SD 0.017; n = 5), respectively at a pH\(^{\text{ex}}\) = 7.15. Thus, in the absence of HCO\(_3^-\), the pH\(^{\text{cyt}}\) values are significantly higher in microvascular than in macrovascular endothelial cells (p < 0.05). Because pH\(^{\text{ex}}\) may affect pH\(^{\text{cyt}}\) regulation, we performed experiments in the presence and absence of HCO\(_3^-\) in microvascular and macrovascular endothelial cells at various pH\(^{\text{ex}}\) values (6.5, 7.0, 7.15, and 7.4). The data show that the pH\(^{\text{cyt}}\) values of microvascular endothelial cells are unaffected by HCO\(_3^-\), whereas the pH\(^{\text{cyt}}\) values of macrovascular endothelial cells are ca. 0.15 pH unit higher in the presence than in the absence of HCO\(_3^-\) throughout the entire pH curve (i.e., pH\(^{\text{ex}}\) = 6.5 - 7.4). These data suggest that pH\(^{\text{cyt}}\) regulation is accomplished via distinct mechanisms in microvascular and macrovascular endothelial cells.

**Acid loading experiments allow the identification of Na\(^+\)- and HCO\(_3^-\)-independent pH\(^{\text{cyt}}\) recovery.** To study the differences in the mechanisms of pH\(^{\text{cyt}}\) regulation between microvascular and macrovascular endothelial cells, we selected conditions in which the two main pH\(^{\text{cyt}}\) regulatory mechanisms should be dormant. Thus, acid loading experiments utilizing the NH\(_4\)Cl pre-pulse technique (47) were used to evaluate the characteristics of the pH\(^{\text{cyt}}\) recoveries in the absence of Na\(^+\) and HCO\(_3^-\). Our expectations were that this experimental strategy could reveal a novel Na\(^+\)- and HCO\(_3^-\)-independent pH\(^{\text{cyt}}\) regulatory mechanism. Cells loaded with
SNARF-1 were perfused with CPB, until steady-state pH\textsuperscript{\text{est}} was achieved. The addition of 25 mM NH\textsubscript{4}Cl caused a rapid intracellular alkalinization (Fig 3A), while the acute removal of NH\textsubscript{4}Cl reversed the condition and caused a rapid acidification in both microvascular and macrovascular endothelial cells. In the absence of Na\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-}, macrovascular endothelial cells did not recover from acidification \([\text{dpH/dt} = 0.001 \text{ (SD 0.005; n = 11)}, \text{J}_{\text{H}^+} = 0.01 \text{ (SD 0.1; n = 11)} \text{ mM H}^+/\text{min}]\), but microvascular endothelial cells did recover from this acid load (Fig 3A, 3B). The H\textsuperscript{+} buffering capacities (\( \beta \)) were significantly higher in microvascular than in macrovascular endothelial cells (in mM): \( \beta = 36.7 \text{ (SD 1.08; n = 11)} \) and \( \beta = 30.9 \text{ (SD 1.37; n = 11)} \), respectively \((p < 0.05)\). To determine if the pH\textsuperscript{\text{est}} recovery in microvascular endothelial cells was mediated by V-H\textsuperscript{+}-ATPases, we examined pH\textsuperscript{\text{est}} recovery from acid loads in a Na\textsuperscript{+}- and HCO\textsubscript{3}\textsuperscript{-}-free buffer, in the presence of bafilomycin, to inhibit V-H\textsuperscript{+}-ATPase. This resulted in a significant decrease in the \( \text{J}_{\text{H}^+} \) (Fig 3B). P-type H\textsuperscript{-}-ATPase inhibitors such as SCH 28080, had no effect on the \( \text{J}_{\text{H}^+} \) (Fig 3B). To determine if Na\textsuperscript{+}/H\textsuperscript{+} exchange and HCO\textsubscript{3}\textsuperscript{-}-based H\textsuperscript{+} transport contributed to pH\textsuperscript{\text{est}} regulation in microvascular endothelial cells, we performed acid loading experiments in the presence of Na\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-}. The \( \text{J}_{\text{H}^+} \) were similar to those observed in media containing Na\textsuperscript{+} and lacking HCO\textsubscript{3}\textsuperscript{-} (cf. Fig 3B). In the presence of Na\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-}, macrovascular endothelial cells also recover from an acid load \([\text{J}_{\text{H}^+} = 1.44 \text{ (SD 0.34; n = 5)} \text{ mM H}^+/\text{min}]\). These \( \text{J}_{\text{H}^+} \) are similar to those observed in microvascular endothelial cells. Collectively, these data indicate that both microvascular and macrovascular endothelial cells exhibited the ubiquitous Na\textsuperscript{+}- and HCO\textsubscript{3}\textsuperscript{-}-dependent pH\textsuperscript{\text{est}} regulatory mechanisms. Importantly, microvascular, but not macrovascular endothelial cells exhibited an additional Na\textsuperscript{+}- and HCO\textsubscript{3}\textsuperscript{-}-independent pH\textsuperscript{\text{est}} regulatory system that allowed them to better cope with acid loads (cf., Fig 3A, 3B). To further demonstrate that
neither Na⁺/H⁺ exchanger nor HCO₃⁻-based H⁺-transporting mechanisms were involved in the observed pH⁹³ recovery, we performed experiments in the absence of Na⁺- and HCO₃⁻ with 5- (N,N-hexamethylene)-amiloride (HMA) and DIDS, blockers of Na⁺/H⁺ exchanger and anion transport, respectively (Fig 3B). Neither HMA (not shown) nor DIDS significantly alter the kinetics of pH⁹³ recovery observed in a Na⁺- and HCO₃⁻-free buffer. These data indicate that microvascular endothelial cells exhibit a Na⁺- and HCO₃⁻-independent pH⁹³ regulatory mechanism to recover from acid loads that is absent in macrovascular endothelial cells.

**Na⁺ removal elicits a transient pH⁹³ decrease in microvascular endothelial cells.** Cell types that exhibit the Na⁺/H⁺ exchanger as a major pH⁹³ regulatory system respond to acute Na⁺ removal (in the absence of HCO₃⁻) with either a rapid or a slow decrease in pH⁹³ (68). In the absence of Na⁺ and HCO₃⁻, most cells do not recover from this acidification (27). This is the case for macrovascular endothelial cells that respond to Na⁺ removal with a slow acidification [ΔpH⁹³ = 0.28 (SD 0.03; n = 6) pH unit; t₁/₂ of acidification = 163 (SD 30; n = 6) sec; Fig 4A]. Interestingly, Na⁺ removal in microvascular endothelial cells resulted in a rapid acidification [ΔpH⁹³ = 0.17 (SD 0.03; n = 11) pH unit] followed by a rapid recovery to baseline levels (Fig 4A). This recovery occurred in a HCO₃⁻-free buffer and was unaffected by preincubation with DIDS (Fig 4B). Importantly, the pH⁹³ recovery in a Na⁺- and HCO₃⁻-free buffer was decreased by bafilomycin A₁, a V-H⁺-ATPase inhibitor (Fig 4B). These data suggest that the V-H⁺-ATPase was responsible for the pH⁹³ recovery from an acid load in microvascular endothelial cells and that Na⁺/H⁺ exchange is the likely pH⁹³ regulatory mechanism used by macrovascular endothelial cells.
Spectral imaging microscopy shows that the leading edge exhibits a more alkaline pH\textsuperscript{cyt} than the lagging edge of the cell in wounded monolayers. Since microvascular but not macrovascular endothelial cells exhibit pmV-ATPase as a unique pH\textsuperscript{cyt} regulatory mechanism, we focused on microvascular endothelial cells to evaluate if there is a distinct pH\textsuperscript{cyt} regulation in domains exhibiting pmV-ATPase. These studies were performed in wounded monolayers (cf. Fig 1C). We used this approach because after wounding, the cells move forward to close the wound, thus creating a polarized system where the leading edge is at the wound site and the lagging edge is at the cell’s rear. 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 cells exhibiting a distinct pH\textsuperscript{cyt} gradient from leading to lagging edge of the cell (cf. Fig 2). To eliminate any bias in our interpretation of the data regarding differences in pH\textsuperscript{cyt} (that could be due to differences in dye concentration and/or intracellular environment that may be distinct at the leading [LEAD] or the lagging [LAG] 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\textsuperscript{+} 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. The insert (box) shows the selected cell where the leading domain is the wounded side and the lagging edge is the cell’s rear (Fig 5B). Decreasing the slit width from 2000 μm (Fig 5B) to 1000 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 5A-5C). Figure 5E shows the first order spectra of Fig 5D. For these experiments, we binned 15 regions of interest (ROI), each corresponding to ca. 5 μm across the length of the cell from leading to lagging edge. For purposes of data presentation, only 2 spectra are shown, corresponding to the leading and lagging edge (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 in the leading than in the lagging edge by ca. 0.2 pH unit (cf. Fig 5F). The salient spectral properties of SNARF-1 show the predicted behavior for this ratiometric dye: increases and decreases in the fluorescence signal at 644 and 584 nm, respectively as pH is increased. The pH gradient more alkaline at the leading than at the lagging edge of the cell is sustained (Fig 5G). The NH₄Cl treatment elicits a cytosolic alkalinization and its removal a cytosolic acidification in the absence of Na⁺ and HCO₃⁻. The magnitude of the pH⁰⁰⁰ changes following NH₄Cl treatment and its removal were larger in the lagging than in the leading edge, consistent with lower H⁺ buffering capacity in the lagging edge of the cell.

The validity of these estimations on pH⁰⁰⁰ values rely on the ability to fully collapse the pH⁰⁰⁰ gradients across all compartments. We have performed complete in situ titrations at discrete distances of the cell from leading to lagging edge (i.e. at ca.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 (i.e. viscosity, protein binding, etc.) that may exist in discrete cellular regions from leading to 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 the pKₐ, of the dye in any of the regions.
studied, indicating that the pH$_{\text{cyt}}$ gradients were fully collapsed (Fig 6B). There are however, significant differences in the $R_{\text{max}}$ and $R_{\text{min}}$ (Fig 6C). Altogether, the data indicate that the distinct pH$_{\text{cyt}}$ values observed in the leading and lagging edge are due to distinct pH$_{\text{cyt}}$ 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 pH regulation. This was demonstrated by pharmacological and ion substitution experiments. Thus, pmV-ATPase is a novel mechanism that allows microvascular endothelial cells to cope with the acidic extracellular environment. Recent studies have shown that V-H⁺-ATPases play a crucial role in growth and phenotypic modulation of myofibroblasts that contribute to neointimal formation in cultured human saphenous vein (38). This process also requires extensive vascular remodeling and involves several cell types, including myofibroblasts, fibroblasts, smooth muscle cells, and endothelial cells (38). In that study, it was shown that the 16 kDa subunit of the V-H⁺-ATPase was overexpressed predominantly in the perinuclear region of the myofibroblasts. The 16 kDa subunit is considered to be the principal component of the V₀ membrane channel sector of the V-H⁺-ATPase, which is located predominantly in acidic organelles, lysosomes and the trans-Golgi network (36). The main function of the V-H⁺-ATPase in these organelles is the maintenance of an acidic pH, 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 play a role in regulating pH in the cytosol, in addition to its well recognized role in regulating the acidity of intracellular organelles.

Migration and invasion through extracellular matrix are important elements in angiogenesis, we therefore evaluated if 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 that do not exhibit pmV-ATPase. Because vascular remodeling following a lesion involves migration of endothelial cells from the non-injured 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 cells migrating towards the damaged region to repair it (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 ca. 24 hours. Importantly, bafilomycin treatment significantly decreases 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 microvascular and macrovascular endothelial cells, we evaluate this parameter. Our data indicated that the doubling times in microvascular endothelial cells is ca. 27 hrs, whereas in macrovascular endothelial cells is ca. 40 hrs. Since microvascular endothelial cells close the wound in 24 hrs, and the doubling times in microvascular and macrovascular endothelial cells are greater than 24 hrs, this supports our contention that the different rates of wound closure are not due to differences in cell growth between microvascular and macrovascular endothelial cells, but rather to distinct migratory rate between these cell types.

The suggestion that microvascular endothelial cells exhibit faster rates of wound closure than macrovascular endothelial cells has been previously reported (3). It has been estimated that human microvascular endothelial cells migrate at ca. 15 μm/hr whereas HUVEC cells migrate at
ca .10 μm/hr under non-stimulating conditions (3). There are however controversies regarding
rates of cell migration. Specifically, it has been shown that wounded monolayers of HUVEC
exhibit 27% wound closure within 24 hrs (33). In this case, the wound size was ca. 2.5 mm.
However, faster migration rates in wounded monolayer in HUVEC and human coronary aortic
endothelial cell, where wound closure is ca. 40% and 30%, respectively within a period of 6 hrs
have also been reported (2). The wound size in these other studies was ca 150-200 μm. Thus, it
appears that a major reason for the different rate of migration is wound size, since larger wounds
are associated with slower rates, possibly due to the release of chemoattractants from cells that
work in a paracrine fashion, stimulating cell migration. It is possible that distinct rates of wound
closure between microvascular and macrovascular endothelial cells may be due to distinct
sensitivity of microvascular endothelial cells to chemokines (59). Microvascular endothelial cells
from lung and kidney produce more chemokines such as fractalkine, interleukine-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, bFGF and IL1β than HUVEC (59). Significant cell division as a mechanism of wound healing is
not likely to occur in cells before 15-24 hrs (2, 25), since endothelial cells exhibit slower doubling
times. Thus, our study extend 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 observed in microvascular endothelial cells when compared to
macrovascular endothelial cells.

Our observations that bafilomycin decreases the rate of cell migration are in agreement
with a recent study that showed that higher concentrations of bafilomycin (ca. 100-500 nM) than
those used in our study, suppressed cell motility in NIH3T3 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, 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 16kDa 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 16 kDa subunit being overexpressed was not evaluated in that study, these data indicate that overexpression of V-ATPase may be important for invasion. Our immunocytochemical data shows that V-ATPase co-localizes 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 co-localizes with actin at the cell’s cortex in the ruffle border of activated osteoclasts (21, 24), and in the apical region of the Manduca’s middle gut epithelium (62). In human breast cancer cells, we have recently shown 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.

The pH\textsuperscript{extracisternal} regulation in most cells is accomplished by the relative contribution of Na\textsuperscript{+}/H\textsuperscript{+} exchanger and HCO\textsubscript{3} \textsuperscript{-}-based H\textsuperscript{+}-transporting mechanisms (18, 44, 47). Microvascular endothelial cells are not the exception, since they exhibited both Na\textsuperscript{+}/H\textsuperscript{+} exchanger and HCO\textsubscript{3} \textsuperscript{-}-based H\textsuperscript{+}-transporting mechanisms. In addition to these important pH\textsuperscript{extracisternal} regulatory mechanisms, pmV-ATPases are also used to regulate pH\textsuperscript{extracisternal} in microvascular, but not in macrovascular endothelial cells. Furthermore, wounded monolayer experiments to reveal the leading edge of migrating cells,
indicated that pmV-ATPase is present at the leading edge. As a result, cells exhibit a more alkaline pH at the leading than at the lagging edge. Differences in pH regulation in the leading and lagging edge are predicted by flux ratio equations since the passive H⁺ influx is ca. 45 and 56 times the passive efflux at the leading edge and lagging edge, respectively (assuming a ψ = -90 mV; and using the pH values shown in Figure 5G for leading and lagging edge, at a pH = 7.4). This suggests that H⁺ influx is larger in the lagging edge, consistent with a more dynamic pH regulatory system at the leading edge. Further support for a dynamic mechanism to maintain such pH differences in leading and lagging edges is based on the fact that although the H⁺ ion permeability is extremely high (P_H⁺ = 10⁻³ cm/sec), the actual J_H⁺ across the plasma membrane is very low because of the low free [H⁺] in the cytosol and in the extracellular environment (if we assume a pH = 7.4). Under these conditions, the passive H⁺ ion influx is ca. 0.02 pH unit/hr, yet the observed differences in pH between the leading and lagging edge are ca. 0.2 pH unit within the time frame of our experiments (i.e. 5-20 msec). Thus, it is unlikely that such differences in pH values from leading to 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 regulatory system that allows these pH gradients to exist in microvascular endothelial cells.

The variation in the steady-state deprotonated/protonated SNARF-1 ratio may be due to actual pH differences, to variations in the regional cytoplasmic microviscosity (28, 43, 55, 66), or even due to different proportion of dye bound to cytoplasmic proteins (4). To properly interpret the differences in SNARF-1 protonated/deprotonated ratios, we have taken into account the behavior of the pH fluoroprobe in the cytoplasm, since it is heterogeneous in terms of composition and organization. Regional intracellular microenvironments may differ in viscosity,
that in turn could result in distinct behavior of the fluoroprobes (39). Indeed, it is known that ion-sensitive fluoroprobes may display not only spectral differences between in vitro and in situ environments (7, 42), but also within the distinctive intracellular organelles (1, 12, 40, 56).

Viscosity values for the leading edge (3.8 mPas), lagging edge (0.5 mPas) 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 the regional variations in ratio, and the in situ calibration parameters (pK_a’, R_{max}, R_{min}) used to estimate pH_{cyt}.

Important effects of protein on the in vitro calibration parameters have been described for several ion indicators, including pH fluoroprobes (4, 41, 54). However, it has also been suggested that SNARF-1 does not bind to bovine serum albumin (BSA), but that a contaminant present in the commercially available SNARF-1 binds to BSA (67). Furthermore, in the cytoplasm of cardiac myocytes, a major fraction of the fluoroprobes (0.5 to 0.9) appears to be bound to proteins (4). Other variables that could cause variations in the in situ titration parameters include: partition of the dye between cytoplasm and endomembranous compartments (37, 61), amount of dye bound to proteins, quenching agents, and inner filter phenomena (7, 53). The reasons for the distinct in situ calibration parameters in different cellular domains are not immediately apparent. However, because the cytoplasmic microenvironment is different in terms of protein composition and viscosity, this could cause distinct diffusion mobility of fluorescent probes and distinct spectral properties. Indeed, studies by fluorescence recovery after photobleaching (FRAP) have shown that the translational diffusion of intracellular BCECF near the membrane and in the bulk cytoplasm is 6-10 times and ~ 4 times lower than in water, respectively (55). However, the fluid-
phase cytoplasmic viscosity in the absence of collisions or binding to cytoplasmic macromolecules is similar to the viscosity of water (26, 55). There are also important differences in molecular crowding within the different subcellular compartments, suggesting considerable diffusional heterogeneity for small metabolites, and thereby fluoroprobes, within different intracellular organelles (14). In addition, viscosity can alter the spectra of ion indicators (28, 43, 55).

To compensate for the cytoplasmic microenvironment differences in terms of viscosity and protein environment, we converted subdomain fluorescence ratios to pH\textsuperscript{cyt} using specific regional calibration parameters (pK\textsubscript{a}', R\textsubscript{max}, and R\textsubscript{min}). These data indicate that the heterogeneities in regional pH\textsuperscript{cyt} values are associated with physiological pH\textsuperscript{cyt} differences, where the leading edge exhibits a more alkaline pH\textsuperscript{cyt} than the lagging edge.

To conclude, our data indicate that pmV-ATPase expression in microvascular endothelial cells is relevant for pH\textsuperscript{cyt} regulation and migration. It is important to note that pmV-ATPases have also been found in highly invasive tumors (51). Thus, pmV-ATPase has physiological significance and could provide a target for pharmacological intervention in angiogenesis and cancer.
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LITERATURE CITED


4. **Baylor SM, and Hollingworth S.** Measurement and interpretation of cytoplasmic 
\[Ca^{2+}\]


8. **Bowman EJ, Siebers A, and Altendorf K.** Baflomycins: a class of inhibitors of membrane


39. **Owen CS.** Comparison of spectrum-shifting intracellular pH probes 5'(and 6')-carboxy-10-
dimethylamino-3-hydroxyspiro[7H-benzo[c]xanthene-7, 1'(3'H)-isobenzofuran]-3'-one and

40. Perez-Terzic C, Stehno-Bittel L, and Clapham DE. Nucleoplasmic and cytoplasmic
differences in the fluorescence properties of the calcium indicator Fluo-3. Cell Calcium

41. Perrin DD, Dempsey B. Buffers for pH and Metal Control. Chapmand and Hall Ltd., 176 pp,
1974.

42. Petr MJ, and Wurster RD. Determination of in situ dissociation constant for Fura-2 and
quantitation of background fluorescence in astrocyte cell line U373-MG. Cell Calcium

43. Poenie M. Alteration of intracellular Fura-2 fluorescence by viscosity A simple correction.

44. Putnam RW. Intracellular pH regulation. In: Speralakis N. editor: Cell Physiology Source

45. Reddy A, Caler VE, and Andrews NW. Endosomes and wound healing plasma membrane

46. Reuveni M, Evenor D, Artzi B, Perl A, and Erner Y. Decrease in vacuolar pH during
petunia flower opening is reflected in the activity of tonoplast H\(^{+}\)-ATPase. J. of Plant
Physiol. 158; 991-998, 2001


48. Selden SC, and Schwartz SM. Cytochalasin B inhibition of endothelial proliferation at


56. Thomas PJ, Gaspers LD, Pharr C, and Thomas JA. Continuous measurement of


64. Wu Y, Liao X, Wang R, Xie XS, and De Brabander JK. Total synthesis and initial


Figure Legends

Figure 1.

(A) Invasion of micro- and macrovascular endothelial cells. Cells (5 ×10^4) were plated onto extracellular matrix protein-coated polycarbonate filters (Osmonics Corp., Minnetonka, MN) containing 10 μm pores in a modified Boyden chamber. Cells were incubated for 24 hours at 37°C. The invading cells were then removed with 2 mM EDTA/PBS, fixed, stained and visually counted. Data are expressed as mean ± S.E.M. (n = 4) *p < 0.05 microvascular versus macrovascular. (B) Migration of micro- and macrovascular endothelial cells. Cells were handled as in (A), except that the inserts were not coated. Cell migration was determined as in (A). Data are expressed as mean ± S.E.M. (n = 4). *p < 0.05 microvascular versus macrovascular. (C-D) Wounded monolayer model allows to study migration in micro- and macro-vascular endothelial cells. Cells were grown onto coverslips and at confluency, a 300 μm wound was inflicted on the confluent cell monolayer. Cells were then fixed, permeabilized, and stained with FITC-phalloidin at either t = 0, or after 18 hours. Confocal microscopy images were obtained with a 20x objective and the analysis of migration was performed on 3 different fields per coverslip. (E- F) Quantification of migration of wounded monolayers of micro- and macro-vascular endothelial cells in the absence or presence of bafilomycin. Data were derived from experiments similar to those shown in (C-D). Data are expressed as mean ± S.E.M. (n = 5). *p < 0.05 when compared to bafilomycin-untreated.
Figure 2.

Immunocytochemical localization of V-ATPase in microvascular (A-C) and macrovascular (D-F) endothelial cells. Cells were grown onto glass coverslips and at confluence, cells were wounded as described in Fig 1 C. 12-16 hrs thereafter cells were fixed and permeabilized. Cytoskeleton was labeled with Alexa fluor-488-phalloidin (A, D). The A subunit of V-ATPase is shown in red (B, E). Sequential images of Alexa-phalloidin 488 (green) and Alexa fluor 568 (red) fluorescence are obtained with a Zeiss LSM 510/META confocal microscope (Ar2/HeNe1 at 30 mW and 1 mW, respectively; Ex = 488 and 543 nm; Em = 515 and 585 nm) using a 60 x objective. Merge images of red (V-ATPase) and green (actin) were assessed on a pixel by pixel basis using Physiology image software V 3.0 (Zeiss) (C, F). Notice that pmV-ATPase is conspicuous in microvascular endothelial cells, and incospicuous in the plasma membrane of macrovascular endothelial cells (long arrows)

Figure 3.

(A) Micro-but not macro-vascular endothelial cells exhibit a Na⁺- and HCO₃⁻-independent pH⁹⁹ regulatory system. Cells were grown on glass cover slips to confluency and intracellularly loaded with SNARF-1 AM and transferred to the spectrofluorometer for pH⁹⁹ measurements. Cells were superfused with CPB until steady-state pH⁹⁹ was reached, then superfusate was exchanged with 25 mM NH₄Cl at the time indicated by the first arrow. Then, the superfusate was exchanged for Na⁺- and HCO₃⁻-free CPB (second arrow). Data are representative of 34 and 11 experiments for micro- and macrovascular endothelial cells, respectively. (B) Effect of inhibitors of primary and secondary H⁺ transport systems on JH⁺ in microvascular endothelial cells.
Cells were handled as described for (A). At the time indicated by the second arrow in (A), the superfusate was exchanged with Na$^+$- and HCO$_3^-$-free CPB containing bafilomycin 50 nM (n = 6), DIDS 100 µM (n = 3); or SCH 28080 5 µM (n = 5). The pH$^\text{in}$ recoveries ($J_{\text{H}^+}$) following acid loading were determined during the first 3 min from experiments similar to those shown in (A). The $J_{\text{H}^+}$ values were obtained by multiplying the $\text{dpH/dt}$ in the first five minutes of recovery by the apparent intrinsic buffering capacity ($\beta_i$) as described earlier (17). Data are expressed as mean ± S.E.M. $^*p < 0.05$ $J_{\text{H}^+}$ when compared to Na$^+$- and HCO$_3^-$-free media.

Figure 4.

(A) **Effect of acute Na$^+$ removal in micro- and macro-vascular endothelial cells.** Cells were handled as in Fig 3A, except that at the arrow, perfusate was changed to Na$^+$-free CPB. Data are representative of 11 and 6 experiments for micro- and macro-vascular endothelial cells, respectively. Notice that micro- but not macro-vascular endothelial cells recover from the Na$^+$-induced acidification.  

(B) **Effect of inhibitors on $J_{\text{H}^+}$ in microvascular endothelial cells.** The pH$^\text{in}$ recoveries following Na$^+$-removal ($J_{\text{H}^+}$) were estimated during the first 3 min from experiments shown in (A). Data are expressed as mean ± S.E.M. $^*p < 0.05$ when compared to Na$^+$-free media. DIDS = 100 µM (n = 3); bafilomycin 50 nM (n = 5).

Figure 5.

**Spectral imaging microscopy to study pH$^\text{ext}$ in leading and lagging edge of the cell.** Microvascular endothelial cells were grown on glass cover slips to confluency and loaded with SNARF-1.  

(A) A single cell is aligned onto the entrance slit (2.0 mm) of the spectrograph,
allowing identification of its leading and lagging edge. (B) Slit width is decreased to (0.5 mm); and then to 0.2 mm (C), to allow for increased spatial resolution and increased signal/noise ratio. (D) Emission filters are removed and the fluorescence spectra are collected and de-convoluted from individual tracks. (E) The emission spectra are collected and recorded from two distinct tracks in the cell (LEAD and LAG; arrows). (F) Spectral pH\textsuperscript{3\textdegree} changes of SNARF-1 from a single track with changes in superfusate: CPB (steady-state), NH\textsubscript{4}Cl (alkalinization), Na\textsuperscript{+}, and HCO\textsubscript{3}\textsuperscript{-}-free CPB (acidification). (G) Recovery from an acid load in Na\textsuperscript{+} & HCO\textsubscript{3}\textsuperscript{-}-free CPB.

Spectra similar to those shown in (F) were collected from 12 different tracks at 50 msec sampling rates. For data presentation only information from the leading (LEAD) and lagging (LAG) edge are shown. At the first arrow, cells were superfused with CPB 25 mM NH\textsubscript{4}Cl and at the time point indicated by the second arrow the superfusate was exchanged for Na\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-}-free CPB.

**Figure 6. In situ calibration of twelve individual tracks from single cells.** Microvascular endothelial cells were handled as described in Figure 5. (A) At the end of the experiment, in situ titrations were performed. The full SNARF-1 spectra were collected at 12 different regions of the cell from leading to lagging edge and the observed ratio values plotted. Only data from 6 tracks are shown. These data were fit into Eqn 1, which was solved iteratively using nonlinear least squares analysis, and the values of pK\textsubscript{a} (B), R\textsubscript{min} and R\textsubscript{max} (C) were determined and plotted as a function of distance from leading to lagging edge of the cell.
Figure 1; Rojas et al.
Figure 3; Rojas et al.
Figure 6; Rojas et al.