pH microdomains in oligodendrocytes

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Summary

Oligodendrocytes (OLs) are cells that produce myelin in the CNS. Here we use ratiometric pH-indicator dye to analyze intracellular pH in OLs in culture. The results reveal alkaline microdomains, which predominate in the perikaryon and proximal dendrites, and acidic microdomains, which predominate in distal dendrites. Spatial nonuniformity of pH is generated by differential subcellular distribution of: Na$$^+$$/H$$^+$$ exchanger (NHE), which is localized in a punctate distribution in the perikaryon and proximal processes, Na$$^+$$/HCO$$^\text{3}\text{-}$$ cotransporter (NBC), which is localized in a punctate distribution in distal dendrites, and CAII, which is colocalized with either NHE or NBC. Inhibition of NHE activity by amiloride inhibits regeneration of alkaline microdomains after cytoplasmic acidification, whereas the inhibition of CAII activity with ethoxyzolamide inhibits acidification of dendrites. Fluorescence correlation spectroscopy (FCS) analysis of CAII microinjected into OLs reveals freely diffusing protein throughout the cell as well as protein associated predominantly with NHE in the perikaryon and predominantly with NBC in the dendrites. Alkaline and acidic microdomains could be generated by transport metabolons consisting of CAII associated with NHE or NBC, respectively. This study provides the first evidence for pH microdomains in cells and describes a mechanism for how they are generated.
Introduction

Intracellular pH is an important modulator of cell function. Many enzymes exhibit pH dependence in the physiological range such that their activities are affected by small variations in intracellular pH. Until recently, intracellular pH was assumed to be spatially uniform because of fast diffusion of H+ ions and buffers. However, recent studies on cytoplasmic pH in epithelial cells, enterocytes, and myocytes indicate that H+ ions diffuse much more slowly in cytoplasm than in buffer (1-3), which means that localized generation or depletion of H+ ions within the cell can result in spatial nonuniformity of intracellular pH (pHi). This could cause intracellular variations in activities of pH-sensitive enzymes.

Oligodendrocytes (OLs) are glial cells that make spiral myelin sheaths around axons in the CNS. The large, flat, extended morphology of OLs in culture facilitates visualization of spatial nonuniformity of intracellular pH. In mature OLs intracellular pH is predominantly determined by activities of three proteins: amiloride–sensitive Na+/H+ exchanger (NHE), ethoxyzolamide-sensitive carbonic anhydrase isotype II (CAII) and stilbene-insensitive Na+/HCO3− cotransporter (NBC) (4, 5). A DIDS-sensitive Cl−/HCO3−exchanger found in oligodendrocyte precursor cells is not present in mature oligodenrocytes (5). Plasmalemmal NHE exchanges extracellular Na+ for intracellular H+ at 1:1 stoichiometry, depleting the cell of H+, thereby causing cellular alkalization (6, 7). Of the seven NHE isoforms, NHE1 appears to be the predominant form responsible for pH homeostasis in the CNS. Non-uniform distribution of NHE has been observed between the apical and basolateral membranes of proximal tubule epithelial cells (8), but the subcellular distribution of NHE in OLs has not been reported. In bicarbonate-free buffer, pHi recovery following an acid load requires external Na+ and is
inhibited by the NHE inhibitor amiloride, indicating that under these conditions NHE is required for cellular alkalinization. Carbonic anhydrase (CA) catalyzes the conversion of CO$_2$ and H$_2$O into HCO$_3^-$ and H$^+$. Among 14 different CA isozymes, carbonic anhydrase II (CAII) is the predominant isozyme found in the brain, where it is concentrated in the myelin compartment of OLs (9-12). Electrogenic Na$^+$/HCO$_3^-$ cotransporter (NBC) exports Na$^+$ and HCO$_3^-$, with a stoichiometry of 1:3 in mature OLs (4). Several NBC isoforms are expressed in mammalian CNS. A characteristic acidic cluster of amino acids (DNDD) in the cytoplasmic C terminal region of NBC mediates association with the basic N-terminal region of CAII creating a functional complex or “transport metabolon” to facilitate HCO$_3^-$ export from the cell (13-16). Similar acidic regions in the cytoplasmic C terminal region of NHE may also mediate interaction with CAII creating a transport metabolon to facilitate H$^+$ export, particularly under acidic conditions (17).

Here we show that OLs in culture contain spatially restricted regions of alkaline and acidic pH (pH microdomains) generated by differential subcellular distribution and colocalization of NHE, NBC and CAII in transport metabolons. Alkaline microdomains predominate in the perikaryon and acidic microdomains predominate in the dendrites. This may provide a mechanism for differential regulation of pH sensitive enzyme activities in different subcellular compartments.
Experimental Procedures

Cells and reagents

Mouse or rat oligodendrocytes were isolated from mixed primary brain cell cultures and grown as previously described (18). SNAFL-calcein acetoxymethyl ester, nigericin, N-isopropyl-N-methylamiloride hydrochloride, BODIPY FL amiloride, DiI, Texas Red dextran, Alexa Fluor monoclonal antibody labeling kit and Alexa-labeled secondary antibodies were obtained from Molecular Probes (Eugene, OR). Texas Red-conjugated and fluorescein-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Ethoxyzolamide and CAII protein from bovine erythrocytes were obtained from Sigma Chemical Co (St. Louis, MO). Rabbit anti-CAII antibody was provided by Dr. Wendy Cammer (Albert Einstein College of Medicine, Bronx, NY) or obtained from Chemicon (Pittsburg, PA). Rabbit antibody raised against peptide 990-1035 of the rat kidney form of NBC-1 was purchased from Chemicon. Mouse monoclonal antibody against peptide 682-801 of rat NHE-1 isoform was obtained from BD Transduction Laboratories (Mississauga, ON). Protein A agarose beads and Polyvinylidene difluoride (PVDF) membranes were from Bio Rad (Hercules, CA). Enhanced chemiluminescence (ECL) was from Pierce biotechnology (Rockford, IL). Protease inhibitors (PMSF, aprotinin, leupeptin and pepstatin) were obtained from Sigma Chemical Co. (St. Louis, MO).

Measurement of intracellular pH

Intracellular pH was measured using cell-permeant SNAFL-calcein acetoxymethyl ester, a pH indicator with dual excitation maxima at 490/540 nm and dual emission maxima at 535/625
nm. The ratio of emission intensities at 625/535 nm for SNAFL-calcein is pH dependent, increasing as pH increases from 6.4 to 7.6. To measure pH$_i$ in a single cell, OLs were loaded with 10 µM of SNAFL-calcein acetoxyethyl ester for 30-40 min at room temperature in HEPES buffered solution (25 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.8 mM MgCl$_2$·6H$_2$O, 1.8 mM CaCl$_2$ and 5.5 mM glucose, pH 7.3). After dye loading, the cells were rinsed twice to wash out unloaded dye and incubated for 15-20 min in HEPES buffer (pH 7.3). For calibration, KCl was substituted for NaCl in HEPES buffer and pH was adjusted with KOH to 6.4, 6.7, 7.0, 7.3, and 7.6. Calibration measurements were done with dye-loaded cells treated with nigericin (a K$^+$/H$^+$ ionophore) to make cells permeable to H$^+$ in solutions at five different pH values (19, 20). Using appropriate discriminatory filters, dual channel images were collected on Zeiss LSM 410 with a 63 x1.4 NA oil immersion objective (Zeiss, Thornwood, NY). Ratiometric images of 625/535 nm were generated for >5 cells at each pH and the average ratio of intensities was calculated for each pH value. These intensity ratio values were used to generate a pH calibration curve for each experiment. The pH$_i$ values in OLs were determined by converting the intensity ratio values at each pixel to the corresponding pH value based on the calibration curve. The resulting ratiometric image was displayed in pseudocolor.

**Inhibitors**

To examine the effects of NHE inhibitor on pH$_i$, OLs were permeabilized with nigericin (10 µM) for 3-5 minutes and acidified in high K$^+$-HEPES buffer (pH 6.4). The cells were then equilibrated in HEPES buffered solution at pH 7.3 for 10 min in the presence or absence of the NHE inhibitor, N-isopropyl-N-methylamiloride hydrochloride (15 µM) to measure pH$_i$ recovery.
To examine the effects of carbonic anhydrase inhibitor on pH$_i$, OLs were treated with ethoxzolamide (200 nM) for 5 min before imaging intracellular pH.

**Immunoprecipitation and western blotting.**

Oligodendrocytes from four 100mm plates were lysed in 450 µl buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% (v/v) NP-40), containing a mixture of protease inhibitors (in µg/ml: 50 PMSF, 1 aprotinin, 1 leupeptin, 1 pepstatin) and incubated at 4°C for 1 h. Lysate was centrifuged (14,000 x g, 4°C, 30 min). The supernatant was first precleared with 50 µl of washed Protein A agarose beads by rocking at 4° C for 1 h. The beads were then spun down, and the supernatant was split. For western blotting aliquots (10 µl) of total lysate were fractionated by SDS-PAGE and were immunoblotted by anti-CAII antibody, anti-NBC antibody, or anti-NHE1 antibody. Positive controls were 0.5 ng CAII protein or rat brain microsomes. For co-immunoprecipitation, supernatants were incubated overnight at 4° C with 10 µg rabbit anti-CAII polyclonal antibodies or 2 µl rabbit pre-immune serum as a control. Protein A agarose beads were then added and allowed to rock for 1.5 h. The beads were washed four times with 0.5 ml of lysis buffer and then boiled for 8 min in 50 µl SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE on 10 or 8 % polyacrylamide gels, and electrophoretically transferred to PVDF membranes. After blocking the membrane with the TBS-T (140 mM NaCl, 20 mM Tris-HCl, 0.1 % Tween-20, pH 7.6) containing 5% nonfat milk, western blotting was performed with mouse anti-NHE monoclonal or rabbit anti-CAII polyclonal primary antibodies, followed by anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase. Immunoreactive bands were visualized with ECL.
Cell staining, confocal microscopy and ratiometric image analysis

BODIPY-labeled amiloride (2 µM) and DiI (15 µg/ml) were used to label Na⁺/H⁺ exchanger (NHE) and the cell membrane, respectively, in live cells. Rabbit anti-CAII antibody was used to stain CA in fixed cells. Texas Red-conjugated dextran (MW <10,000), which diffuses uniformly throughout the cytoplasm and the nucleoplasm, and thus serves as a cytoplasmic volume marker to normalize CAII concentrations in different regions, was microinjected into the cells prior to fixation. The Na⁺/HCO₃⁻ cotransporter (NBC) was stained using rabbit anti-NBC antibody and Alexa 488-labeled donkey anti-rabbit secondary antibody. Dual channel images were collected using a Zeiss LSM 410 confocal microscope with a 63 x 1.4 NA oil immersion objective with appropriate discriminatory filter sets. Neutral density attenuation was used to ensure that all regions of the image were within the dynamic range of the system. To determine the relative concentrations of NHE, NBC, and CAII in different subcellular compartments, ratios of intensities in the green and red channels (NHE/ DiI, CAII/ Texas Red dextran, NBC/ DiI ) were calculated and displayed in pseudocolor using NIH image software, version 1.62 (National Institute of Health).

Colocalization analysis

For CAII/NHE double labeling, rabbit polyclonal anti-CAII and mouse monoclonal anti-NHE primary antibodies were used, followed by Texas Red-labeled donkey anti-rabbit and Alexa 488-labeled donkey anti-mouse secondary antibodies. For CAII/NBC double labeling, CAII protein (100 µg/ml) was labeled with Alexa 647 using an Alexa Fluor monoclonal antibody labeling kit from Molecular Probes. Alexa 647-labeled CAII (10 µg/ml) was microinjected into the OLs followed by fixation with 3.7% formaldehyde. NBC was stained as above. Dual channel images were collected using a Zeiss LSM 510 confocal microscope with a 63 x1.4 NA oil
immersion objective with appropriate discriminatory filter sets. To analyze individual puncta, uniform staining was subtracted in both green and red channels using Photoshop (Adobe). Several hundred individual, well-resolved puncta were selected in both channels and the intensities of CAII and NHE or NBC associated with each punctum were measured as described previously (21) and plotted in a scatter diagram.

**Fluorescence correlation spectroscopy analysis**

To measure intracellular concentrations and diffusion times for CAII in OLs, CAII protein was fluorescently labeled with Alexa 647, using Alexa Fluor monoclonal antibody labeling kit from Molecular Probes, and microinjected into OLs. The FCS observation volume was positioned in either the perikaryon or dendrites. In our FCS system (Zeiss Confocor II), confocal optics is used to define the observation volume (<1 femtoliter) illuminated by a stationary laser beam. Fluorescence fluctuation within the observation volume is detected using low noise avalanche photodiode detectors (APD) with single photon sensitivity. Photons in the observation volume were recorded over 5 seconds and the measurement was repeated five times at each position in the cell. The dynamic properties of CAII molecules at each subcellular location were analyzed by applying the autocorrelation method to the entire fluctuation record (22). The autocorrelation function is calculated by comparing the fluorescence intensity at time (t) to the intensity at time (t + τ). The autocorrelation function for each measurement was computed using Zeiss Confocor software. Autocorrelation curves with significant photobleaching were discarded and the remaining curves for each subcellular location were averaged. The averaged autocorrelation curves were globally fit into the theoretical autocorrelation function for three dimensional diffusion.
Where \( N \) is the number of particles, \( y_{trip} \) is triplet state fraction, \( \tau_{trip} \) is triplet state time, \( CN \) is the total number of diffusion components, \( y_i \) is the fraction of the diffusion component \( i \), \( \tau_i \) is diffusion time for component \( i \), and \( \omega \) is the structural parameter of the observation volume.

Manipulation of raw FCS data and non-linear least squares curve fitting was performed using a system of specialized MATLAB scripts (available upon request). Reciprocals of the standard deviations for each point of the experimental autocorrelation curve were used as weights for fitting.

If fluorescent CAII aggregates or binds to other molecules, and if aggregation or binding alters its diffusion properties, the fraction bound and fraction unbound can be determined. This provides a measure of the diffusion times and proportions for each component. The proportions of each component were converted to absolute concentrations as follows. The total number of endogenous CAII molecules in the cell (~10,000 molecules/cell) was determined by quantitative western blotting (data not shown). The overall concentration of endogenous CAII in the entire cell was estimated based on the total number of molecules per cell and the total volume of the OL (estimated to be ~ 1 pL). The volume fraction of the perikaryon and proximal dendrites was estimated to be approximately equal to the volume fraction of the distal dendrites based on their relative areas and the estimated average thickness of cytoplasm in the two compartments. The average relative concentration of CAII in the perikaryon and proximal dendrites was approximately half that in the distal dendrites, based on quantitative immunofluorescence. This allows estimation of the average absolute concentration of endogenous CAII in the perikaryon and proximal dendrites and in the distal dendrites. Fitting the FCS autocorrelation data provides a
measure of the relative proportions of microinjected fluorescent CAII with different autocorrelation times. Assuming that FCS autocorrelations times for exogenous fluorescent CAII reflect the distribution of endogenous unlabeled CAII, the absolute concentrations of each component in each subcellular compartment can be estimated.
Results

pH microdomains in OLs

OLs were incubated with ratiometric pH indicator dye (seminaphthofluorescein (SNAFL)-calcein acetoxyethyl ester) in bicarbonate-free HEPES buffer (pH 7.3). Following dye loading, dual channel confocal images were collected and converted to ratiometric images to visualize pH$_i$. Calibration was performed with cells permeabilized with nigericin (a K$^+$/H$^+$ ionophore) in high K$^+$-buffer at five different pH values (6.4, 6.7, 7.0, 7.3, 7.6). Nigericin-treated cells showed a uniform distribution of pH throughout the cytoplasm (Fig. 1A shows representative cells at pH 6.4, 6.7, 7.0, 7.3 and 7.6). Ratiometric values were measured in five cells at each pH to generate a calibration curve for intracellular pH (Fig. 1A). Figure 1B shows a representative dual channel image of an OL. The ratios between the channels are not uniform throughout the cell, some regions appear more reddish and some appear more greenish, indicating spatial nonuniformity of pH. The calibration data from Fig. 1A was used to calculate intracellular pH based on the ratios between the two channels at each position in the cell. Figure 1C shows a ratiometric image of the cell in Figure 1B, where intracellular pH is represented in pseudocolor. The perikaryon and some dendrites appear more alkaline (red) while other dendrites appear more acidic (blue). Close examination of the image in (Fig. 1C inset) reveals numerous alkaline microdomains (red) and acidic microdomains (blue) scattered throughout the cell in close apposition to the cell membrane. Microdomains are defined as discrete regions (generally ~ 1 µm in diameter) with pH values that differ from the surrounding cytoplasm by > 0.1 pH unit. In the perikaryon and in some dendrites, alkaline microdomains predominate, making the overall intracellular pH in these regions appear more alkaline. In other dendrites, acidic microdomains predominate, making the overall pH in these regions appear more acidic.
Alkaline microdomains may reflect regions of local proton depletion, and acidic microdomains may represent regions of local proton generation. Spatial nonuniformity in the ratiometric image shown in Fig. 1C is interpreted as evidence for nonuniform intracellular pH. The relatively uniform intracellular pH in nigericin-permeabilized cells used for calibration (Fig. 1A) indicates that spatial nonuniformity of pH in intact cells is not due to nonuniform intracellular dye binding, noise in the imaging system or imaging artifacts.

Subcellular distribution of NHE, CAII and NBC in OLs.

The function of NHE is to export protons from the cytoplasm. Figure 2A shows that NHE-1 is detected in OLs by western blotting. To analyze the subcellular distribution of NHE, OLs were incubated with bodipy-amiloride, which binds to NHE and with DiI, a lipophilic carbocyanine dye, which labels the plasma membrane uniformly (Fig. 2A). Ratiometric images displaying NHE intensity divided by DiI intensity at each pixel provide a measure of the relative concentration of NHE at each point on the cell membrane. NHE is concentrated in a punctate distribution in the perikaryon and proximal processes. Each punctum presumably represents a cluster of NHE molecules formed by association of individual NHE molecules within the plane of the membrane. Since the function of NHE is to exchange extracellular Na\(^+\) for intracellular H\(^+\), punctate distribution of NHE may result in local depletion of H\(^+\) which could create alkaline microdomains in the vicinity of the clusters.

CAII reversibly converts H\(_2\)O and CO\(_2\) to H\(^+\) and HCO\(_3^-\). We have determined, by quantitative western blotting (Fig. 2B), that OLs contain approximately 10,000 CAII molecules per cell (data not shown). To analyze the subcellular distribution of CAII, OLs were microinjected with Texas Red labeled dextran as a cytoplasmic volume marker and then fixed.
and stained with antibody to CAII. The CAII intensity divided by the Texas Red dextran intensity, displayed as a ratiometric image in pseudocolor, provides an indication of the relative concentration of CAII at each position in the cell (Fig. 2B). CAII is distributed at low intensity uniformly throughout the cell and at higher intensity in a discrete punctate distribution in the dendrites. Based on the number of puncta and the number of CAII molecules per cell, each punctum represents 10-100 CAII molecules. The overall concentration of CAII is higher in the dendrites than in the perikaryon, with different concentrations in individual processes.

NBC is a cotransporter that exports intracellular Na\(^+\) and HCO\(_3^-\) from the cell, suggesting that it may play a role in removing HCO\(_3^-\) generated by CAII in the periphery. Figure 2C shows that NBC-1 is detected in OLs by western blotting. To examine the distribution of NBC in OLs, cells were incubated with DiI to label the plasma membrane uniformly and then immunostained with antibody to NBC. The ratiometric image of NBC/DiI displaying the intensity of NBC divided by DiI intensity in pseudocolor provides a measure of NBC concentration at each position on the membrane (Fig. 2C). NBC appears to be localized in a punctate distribution in most dendrites. Each punctum presumably represents a cluster of NBC molecules formed by association of individual NBC molecules in the plane of the bilayer. Since the function of NBC is to export intracellular HCO\(_3^-\), the punctate distribution of NBC may result in local accumulation of H\(^+\) which could create acidic microdomains in the vicinity of the clusters.

**Inhibition of NHE or CAII affects intracellular pH in OLs**

To determine if NHE activity regulates intracellular pH, OLs loaded with SNAFL-calcein were acidified by nigericin treatment at pH 6.4, followed by equilibration in buffer at pH 7.3, in the absence or presence of amiloride to inhibit NHE function (Fig. 3A). Amiloride treatment
inhibited overall pH recovery and also inhibited regeneration of alkaline microdomains, indicating that NHE contributes to establishing and maintaining alkaline intracellular pH in OLs. Since amiloride is known to inhibit several different sodium/proton antiporter isoforms(23, 24), it is possible that other transporters, in addition to NHE, contribute to generating alkaline pH in OLs.

Inhibition of CA with ethoxyzolamide reduces acidification of the dendrites (Fig. 3B), indicating that CA activity plays a role in maintaining acidic pH in the periphery. Because CAII generates both HCO$_3^-$ and H$^+$, it can contribute to acidification of the periphery only if HCO$_3^-$ is removed from the cell.

**Association of CAII and NHE or NBC**

The N terminal region of CAII binds to an acidic cluster containing a characteristic DNDD motif in the C-terminal region of anion exchanger1 (AE1) protein and kNBC1(25, 26). A similar acidic cluster in the C-terminal region of NHE1 may also mediate interaction with CAII (17). Association of CAII with NHE and/or NBC was tested by co-immunoprecipitation. OL extract was immunoprecipitated with antibody to CAII and the immunoprecipitated material was analyzed by western blotting for NHE and NBC. The results in Fig. 4 indicate that CAII interacts with both NHE and NBC. To test the possibility that CAII is colocalized with NHE in the plasma membrane, OLs were double labeled with antibody to CAII and antibody to NHE or NBC. The merged images in Fig. 4 reveal punctate colocalization of CAII and NHE and of CAII and NBC. To perform quantitative colocalization analysis, the uniform staining was substracted in both green and red channels, individual well-resolved puncta were selected, and the intensities in each channel associated with each punctum were measured and plotted in a scatter diagram.
(Fig. 4). 11% of the puncta contained CAII without NHE, 11% contained NHE without CAII, and 78% (129/166) contained both NHE and CAII. Puncta containing only CAII or only NHE had relatively low intensities compared with puncta containing both CAII and NHE, suggesting that the colocalized molecules are present in larger clusters. Furthermore, the intensities of NHE and CAII in each punctum exhibited significant covariation ($r = 0.711$). In other words, clusters with more NHE molecules tended to contain more CAII molecules and clusters with less NHE contained less CAII. This suggests that CAII molecules and NHE molecules are present in approximately stoichiometric amounts in each cluster.

To analyze colocalization between NBC and CAII, OLs were microinjected with Alexa 647-labeled CAII and then immunostained with antibody to NBC. The merged image (Fig. 3) reveals punctate colocalization of CAII and NBC. Quantitative colocalization analysis, performed as before, reveals that 20% of the puncta contained CAII without NBC, 33% contained NBC without CAII, and 47% (83/175) contained both NBC and CAII. Puncta containing only CAII or only NBC had relatively low intensities, whereas puncta containing both molecules had relatively high intensities in both channels, indicating that the colocalized molecules were present in larger clusters. Covariation of CAII and NBC in individual puncta was less pronounced ($r = 0.502$) than for CAII and NHE indicating that individual clusters contain non-stoichiometric amounts of NBC and CAII.

Since CAII can associate with either NHE or NBC, it is possible that some CAII-containing puncta contain both NHE and NBC. However, since NHE is concentrated predominantly in the perikaryon and NBC is concentrated predominantly in the periphery, the proportion of such mixed complexes is likely to be small.
FCS analysis of intracellular CAII

The colocalization results described above identify three distinct pools of CAII in OLs – uniformly distributed throughout the cytoplasm, colocalized with NHE in a punctate distribution in the perikaryon and colocalized with NBC in a punctate distribution in the dendrites. The dynamic behavior of CAII in these different pools was analyzed by fluorescence correlation spectroscopy (FCS), which measures fluorescence fluctuations within a defined observation volume (< 1fl), which can be positioned at different locations within the cell (27). OLs were microinjected with Alexa 647-labeled CAII, and FCS measurements were made at different positions in the perikaryon and in the dendrites for 13 different cells. Autocorrelation analysis (Fig. 6A) was used to determine concentrations and diffusion times ($\tau$) for CAII at each position in the cell. The diffusion time for CAII in buffer was determined to be 163 $\mu$sec, corresponding to a diffusion coefficient, $D$, of $\sim 150 \, \mu m^2/sec$. In both perikaryon and dendrites of OLs, a component with a fast diffusion time ($\tau_1 \sim 426 \, \mu s$) was detected, which presumably corresponds to freely diffusing CAII molecules. The $\sim 2.6$ fold difference in diffusion time between CAII in buffer and in the cytoplasm is probably due to increased viscosity and molecular crowding in the cytoplasm compared to buffer. In addition, two distinct components with slower correlation times ($\tau_2 \sim 2260 \, \mu s$ and $\tau_3 \sim 11620 \, \mu s$) were detected in both the perikaryon and in the dendrites. The absolute concentrations of the different components were estimated based on the total number of CAII molecules in the cell (determined by quantitative western blotting), the relative concentrations of CAII in the perikaryon and dendrites (determined by quantitative immunofluorescence), and the proportions of each component (determined by fitting the FCS autocorrelation data). In the perikaryon, the medium component ($\tau_2 \sim 2260 \, \mu s$) was predominant. In the dendrites the fast component ($\tau_1 \sim 426 \, \mu s$) was predominant and the
concentration of the slow component ($\tau_3 \sim 11620 \mu s$) was greater than the concentration of the medium component ($\tau_2 \sim 2260 \mu s$). Given the differential subcellular distribution and colocalization patterns of CAII, NHE and NBC (Fig 2-4), it is likely that the fast component ($\tau_1 \sim 426 \mu$sec) in both compartments corresponds to uniformly distributed, freely diffusing CAII, while the medium component ($\tau_2 \sim 2260 \mu s$), which predominates in the perikaryon, represents punctate CAII associated predominantly with NHE, and the slow component ($\tau_3 \sim 11620 \mu s$), which is found predominantly in the dendrites, represents punctate CAII associated predominantly with NBC.

There are several possible biophysical explanations for the different correlation times observed by FCS. One possibility is that the medium and slow components represent lateral diffusion of transport metabolon complexes in the plane of the membrane bilayer. Because of the orthogonal orientation of the plasma membrane bilayer in relation to the FCS observation volume, it is difficult to calculate precise lateral diffusion coefficients for CAII/NHE and CAII/NBC transport metabolons based on the measured correlation times. A second possibility is that the medium and slow correlation times reflect association/dissociation of CAII with relatively immobile binding partners, such as NHE and NBC. If the predominant medium component in the perikaryon ($\tau_2 \sim 2260 \mu s$) corresponds to dissociation of CAII from NHE then the off rate ($k_{off}$) for CAII/NHE complexes is $\sim 500/s$. If the predominant slow component in the dendrites ($\tau_3 \sim 11620 \mu s$), corresponds to dissociation of CAII from NBC then the $k_{off}$ for CAII/NBC complexes is $\sim 86/s$. Distinguishing between these possible explanations for the observed FCS correlation times will require further investigation. However, the FCS results do indicate that freely diffusing CAII is found throughout the cell and that the properties of non-freely diffusing CAII in the perikaryon and dendrites are different.
Discussion

The results presented here show that OLs contain alkaline and acidic pH microdomains, and that CAII is colocalized with NHE in the perikaryon and with NBC in the dendrites. Colocalized CAII/NHE and CAII/NBC may constitute “transport metabolons” which could generate the observed pH microdomains, as illustrated in the model in Fig. 7. According to this model, CAII converts CO₂ and H₂O to HCO₃⁻ and H⁺. If CAII is associated with NHE in a transport metabolon, H⁺ ions generated by CAII activity will be more efficiently exported by NHE. Depending on the juxtaposition of their active sites, H⁺ ions generated by CAII activity may be ‘channeled’ directly to NHE for export without exchanging with freely diffusing H⁺ ions. This could result in local depletion of H⁺ ions and accumulation of HCO₃⁻ ions, creating an alkaline pH microdomain in the vicinity of the CAII/NHE transport metabolon. Alternatively, if CAII is associated with NBC in a transport metabolon, HCO₃⁻ ions generated by CAII activity will be more efficiently exported by NBC. Again, depending on juxtaposition of active sites, HCO₃⁻ ions generated by CAII activity may be ‘channeled’ directly to NBC for export without exchanging with freely diffusing HCO₃⁻ ions, which could result in local depletion of HCO₃⁻ ions and accumulation of H⁺ ions, creating an acidic pH microdomain in the vicinity of the CAII/NBC transport metabolon.

Under physiological conditions in the intact animal OLs are buffered by CO₂ and bicarbonate. Previous studies of intracellular pH in OLs compared bicarbonate-containing and bicarbonate-free media (4). In OLs maintained in bicarbonate-containing medium intracellular pH is slightly higher and NBC mediated bicarbonate transport may be close to equilibrium. In OLs maintained in bicarbonate-free medium the intracellular pH is slightly lower and NBC
presumably functions primarily to export bicarbonate produced inside the cell. In this study we used OLs that were grown in a CO₂ incubator in medium containing bicarbonate as well as HEPES buffer. For imaging intracellular pH the cells were transferred to HEPES buffered medium without bicarbonate to minimize pH fluctuations which can occur during extended observation of the cells on the stage of the microscope outside of the CO₂ incubator. Detailed analysis of the effect of CO₂/bicarbonate buffering on pH microdomains in OLs in culture requires careful regulation of the CO₂ atmosphere on the stage of the microscope which is beyond the scope of this study.

The dimensions and pH differentials of alkaline and acidic microdomains in OL dendrites will depend on the relative production rates of HCO₃⁻ and H⁺ ions by CAII, the export rates of H⁺ and HCO₃⁻ ions by NHE and NBC, respectively, and the diffusion rates of HCO₃⁻ and H⁺ ions in the cytoplasm. The production rates of HCO₃⁻ and H⁺ ions are determined by the catalytic activity of CAII in OLs. Because the turnover rate for CAII (~10⁶ s⁻¹) is very fast compared to most other enzymes (28), this is unlikely to be the rate-limiting step for generating pH differentials in microdomains. In contrast, the turnover rates for NHE and NBC are at least three orders of magnitude slower (29, 30), suggesting that NHE activity limits the export rate of H⁺ ions by the CAII/NHE transport metabolon, and NBC activity limits the export rate of HCO₃⁻ ions by the CAII/NBC transport metabolon, thereby limiting the pH differentials of alkaline and acidic microdomains, respectively.

CAII can have different effects on pH microdomains depending on whether the enzyme is freely diffusing or associated with transport metabolons. Freely diffusing CAII will increase the rates of H⁺ and HCO₃⁻ diffusion by generating mobile bicarbonate buffers (31) which will tend to dissipate pH differentials. The apparent diffusion coefficient for H⁺ ions in cytoplasm
(D_{H}^{app} = 37.8 \ \mu m^2 s^{-1}) \ is \ more \ than \ 300 \ times \ lower \ than \ for \ H^+ \ ions \ diffusing \ in \ a \ dilute, unbuffered \ solution \ (3, \ 31) \ presumably \ due \ to \ slowly \ diffusing \ or \ immobile \ buffers \ in cytoplasm. \ However, \ in \ cells \ maintained \ in \ CO_2/\ HCO_3^- -buffered \ conditions, \ the \ diffusion \ coefficient \ of \ H^+ \ is \ 6-fold \ greater \ than \ that \ in \ HEPES (31). \ Therefore, \ since \ intracellular \ CO_2/ HCO_3^- buffering \ is \ mediated \ by \ CAII \ activity, \ the \ intracellular \ mobility \ of \ H^+ \ will \ be \ increased by \ freely \ diffusing \ CAII \ activity. \ This \ will \ tend \ to \ dissipate \ pH_i \ differentials \ by \ providing \ more \ mobile \ buffers. \ On \ the \ other \ hand, \ CAII \ associated \ with \ NHE \ and \ NBC \ transport \ metabolons will \ increase \ local \ concentrations \ of \ HCO_3^- \ and \ H^+, \ respectively, \ thereby \ tending \ to \ increase \ pH differentials. \ Thus, \ the \ pH \ differentials \ and \ dimensions \ of \ pH \ microdomains \ may \ be \ determined, at \ least \ in \ part, \ by \ the \ partition \ coefficient \ between \ freely \ diffusing \ and \ metabolon-associated CAII.

The locations of pH microdomains within the cell are determined by the subcellular distributions of CAII, NHE, and NBC in OLs. Subcellular distributions of specific proteins are often affected by localization of their encoding RNAs. In OLs, RNAs (such as myelin basic protein RNA) that contain a specific 11 nucleotide cis-acting sequence (GCCAAGGAGGCC), termed the hnRNP A2 response element (A2RE), are transported preferentially along microtubules to the distal dendrites by a trafficking pathway mediated by hnRNP A2 (18, 32). Both CAII RNA and NBC RNA contain A2RE-like sequences (ACCAGAGAACA and GCCAAGGAGGCC, respectively), and therefore are likely co-transported by the hnRNP A2 pathway. This could result in localized co-translation of CAII and NBC proteins and formation of CAII/NBC transport metabolons at sites of RNA localization, which could generate acidic microdomains in the distal dendrites of OLs. Conversely, NHE RNA, which does not contain an A2RE-like sequence, and therefore should not be transported to the distal dendrites, will remain
in the perikaryon, resulting in localized synthesis of NHE in the perikaryon and proximal dendrites. NHE localized to the perikaryon and proximal dendrites may recruit soluble CAII into CAII/NHE transport metabolons to generate alkaline microdomains.

While RNA localization can affect the location of pH microdomains, intracellular pH can conversely influence RNA localization by affecting transport along microtubules. Transport of organelles towards the plus ends of microtubules is favored under acidic conditions while transport to the minus ends is favored under alkaline conditions (33-35). If transport of RNA granules on microtubules in OLs exhibits similar pH dependence, acidic microdomains generated by CAII/NBC transport metabolons in the distal dendrites may stimulate further transport of CAII and NBC RNAs towards the plus ends of microtubules in the periphery. This could result in formation of additional CAII/NBC transport metabolons, generating additional acidic microdomains. Thus, acidic microdomains may act as “attractors” for RNA transport. Conversely, alkaline microdomains generated by CAII/NHE transport metabolons will favor the retention of NHE RNA in the perikaryon, resulting in formation of additional CAII/NHE metabolons to generate additional alkaline microdomains. Thus, pH microdomains may represent a positive feedback mechanism to enhance differential RNA localization in OLs.

Neuronal activity-dependent pH fluctuations in the brain interstitial space are accompanied by rapid intracellular pH fluctuations (36-39). Conversely, intracellular pH can influence the electrical activities of both neuronal and glial cells (40). The existence of pH microdomains in OLs may serve to buffer activity-dependent fluctuations in intracellular pH, and may also influence local electrical activity. If OL processes in the CNS contain pH microdomains similar to those seen in culture, these could have complex effects on nerve conduction.
In summary, this paper provides the first description of intracellular pH microdomains in OLs and presents a model for how such microdomains can be generated in the vicinity of CAII/NHE and CAII/NBC transport metabolons. It is likely that such pH microdomains exist in other cell types. However, they may be more easily visualized in oligodendrocytes because of the unique flattened and extended morphology of these cells. The existence of pH microdomains provides a potentially important mechanism for local modulation of intracellular enzyme activities, particularly molecular motor activities in RNA trafficking, and for spatial regulation of neuronal and glial electrical activity.
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Abbreviations

CA II, carbonic anhydrase II; CNS, central nervous system; FCS, fluorescent correlation spectroscopy; NHE, sodium hydrogen exchanger; NBC, sodium bicarbonate cotransporter; OL, oligodendrocyte; SNAFL, seminaphthofluorescein.
References


Figure Legends

**Fig. 1 Intracellular pH in oligodendrocytes**

Panel A – Calibration of pH\(_i\) in OLs. Cells were loaded with 10 \(\mu\)M SNAFL-calcein for 30-45 min in HEPES buffer (pH 7.3) and treated with 10 uM nigericin for 3-5 min in high K\(^+\) (145 mM) -containing HEPES buffers at different pH values (6.4, 6.7, 7.0, 7.3, 7.6). Dual channel confocal images were collected from the green channel (excitation: 488 nm, emission: 525 nm) and the red channel (excitation: 568 nm, emission: 615 nm). Ratio images of red/green channels were generated for each image collected and average cytoplasmic ratio values were calculated for >5 cells at each pH. The values obtained were used to generate a pH calibration curve. Error bars indicate standard deviations of the ratios among >5 cells at each pH value.

Panel B – Dual channel image of SNAFL in intact OL. Cells were loaded with the pH indicator dye as above and rinsed twice to wash out unloaded dye. Dual channel confocal images were collected from intact cells equilibrated in HEPES buffer (pH 7.3). A red/green merged image of a representative cell is shown.

Panel C – Ratiometric image of intracellular pH in OL. Intracellular pH values at each pixel in the image in panel B were calculated from the calibration data in panel A. The intracellular pH values are displayed in pseudocolor with alkaline pH in red and acidic pH in blue color. In the inset, acidic or alkaline pH microdomains are indicated by blue and red arrows, respectively. The scale bar is 10\(\mu\)m.

**Fig. 2 NHE, CA and NBC in OLs**

Panels A-D provide evidence for NHE in OLs. Panel A shows a western blot of NHE-1 in OL lysate. The control lane was loaded with lysate from rat liver microsomes. The positions of molecular weight standards are indicated. Panels B-D show the subcellular distribution of...
NHE-1. OLs were incubated with DiI (15 µg/ml) for 20 min at room temperature to label the cell membrane. BODIPY-labeled amiloride (2µM) was used to label Na⁺/H⁺ exchanger (NHE) protein in OLs by incubating for 10 min. The green channel (B) shows amiloride labeling of NHE and the red channel (C) shows the DiI labeling of the plasma membrane. The single channel images are displayed in inverted grayscale to facilitate visualization of fine processes. The relative concentration of NHE at each pixel was normalized by dividing the intensity in the NHE image by the intensity in the corresponding pixel in the DiI image. The ratiometric image of NHE/DiI (D) is displayed in pseudocolor where the highest NHE concentration is displayed in red, and the lowest in blue.

Panels E-H document CAII in OLs. Panel E shows western blotting of CAII in OL lysate. The control lane was loaded with purified CAII. Panels F-H show the subcellular distribution of CAII in OLs. Texas Red-conjugated dextran (M.W <10,000) was microinjected into the cells as a cytoplasmic volume marker, followed by fixation with 3.7% formaldehyde and immunolabeling with rabbit anti-CAII primary antibodies and fluorescein-labeled donkey anti-rabbit secondary antibodies. CAII and cytoplasmic volume were visualized in green (F) and red (G) channels, respectively, and are displayed in inverted grayscale. The intensity of CAII in the green channel was divided by the intensity of Texas Red dextran in the red channel. The ratiometric image (H) of CAII/cytoplasmic volume was displayed in pseudocolor, where the highest CAII concentration is shown in blue and the lowest in red.

Panels I-L document NBC in OLs. Panel I shows western blotting of NBC-1 in OL lysate. The control lane was loaded with lysate from rat liver microsomes. Panels J-L show the subcellular distribution of NBC-1 in OLs. Cells were fixed and stained with DiI to label the plasma membrane, NBC was immunolabeled with rabbit anti-NBC primary antibody and Alexa
488-labeled anti-rabbit secondary antibody. Panel J and K show the staining of NBC and plasma membrane, respectively, displayed in inverted grayscale. The ratiometric image (L) of NBC/DiI was displayed in pseudocolor, where the highest NBC concentration is shown in blue and the lowest in red. Note that the pseudocolor coding in panels F and I is inverted relative to panel C to facilitate visualization of color variations in the distal dendrites.

**Fig. 3 Effects of NHE and CA inhibitors on intracellular pH in OLs**

SNAFL-calcein-loaded cells were acidified with 10 µM nigericin for 5 min in high K⁺-HEPES buffer (pH 6.4). After acidification, cells were equilibrated in HEPES buffer containing 140 mM NaCl (pH 7.3) for 10 min in the absence (A) or presence (B) of the NHE inhibitor, N-isopropyl-N-methylamiloride hydrochloride (15µM). Ratiometric images of intracellular pH were generated from the emission intensities of red/green channels. Dye-loaded OLs were treated with 200nM ethoxyzolamide, a CA inhibitor, for 5 min followed by ratiometric imaging of intracellular pH (C). The color bar was generated from the standard calibration curve for each set of experiments. White indicates regions outside the calibration range (pH >7.6). The cells shown are representative of three separate experiments with different batches of cells. At least ten cells were analyzed in each experiment. The scale bar is 10 µm.

**Fig 4. Interaction of CAII with NHE and NBC**

Panels A-C document interaction of CAII with NHE. Panel A shows co-immunoprecipitation of NHE-1 with CAII. CAII was immunoprecipitated from OL lysate followed by western blotting with NHE-1 antibody. The NHE-1 band was marked by arrow. Panel B shows colocaliztion of NHE-1 and CAII in OLs. NHE-1 and CAII were double-labeled
with mouse anti-NHE monoclonal and rabbit anti-CAII polyclonal primary antibodies. Alexa 488-labeled and Texas red-labeled secondary antibodies were used to stain NHE-1 (green) and CAII (red), respectively. A representative region containing several dendrites is shown in the merged image, where colocalization of CAII and NHE appears yellow. The scale bar indicates 2 µm. Panel C shows quantitative analysis of colocalization. Uniform staining was subtracted and discrete well-resolved puncta were selected in both channels. The intensities associated with each punctum were calculated for each channel and plotted in a scatter diagram. Covariation between NHE and CAII intensities was calculated as a correlation value, r, between the two channels.

Panels D-F document interaction of CAII with NBC. Panel D shows co-immunoprecipitation of NBC-1 with CAII. CAII was immunoprecipitated from OL lysate and NBC-1 was detected in the immunoprecipitated material by western blotting with NBC-1 antibody. Panel E shows colocalization of NBC-1 and CAII in OLs. Alexa 647-labeled CAII protein (10 µg/ml) was microinjected into OLs and cells were incubated for 15 min at 37 °C to allow equilibration of CAII throughout the cell. Cells were fixed with 3.7% formaldehyde followed by the immunostaining with rabbit anti-NBC primary antibody and Alexa 488-labeled secondary antibody. NBC and CAII were visualized in green and red channels, respectively. A representative region containing several dendrites is shown in the merged image, where colocalization of CAII and NBC-1 appears yellow. The scale bar indicates 2 µm. Panel F shows quantitative analysis of colocalization. Uniform staining was subtracted from the merged image and discrete well-resolved puncta were selected in both channels. The intensities associated with individual puncta were calculated from each channel and plotted in a scatter diagram. The correlation value, r, was calculated for covariation between NBC and CA.
Fig 5. Fluorescence correlation spectroscopy analysis of CAII in OLs

OLs were microinjected with Alexa 647-labeled CAII protein (10 µg/ml) and FCS measurements were taken in the perikaryon and in the dendrite for 13 different cells. Photon count fluctuations in the observation volume were recorded over 5 second intervals and measurements were repeated five times at each point in the cell. Fluorescence fluctuations were analyzed by autocorrelation analysis. Fig 6A shows representative autocorrelation curves for CAII in buffer, in the perikaryon and in the dendrite. The circles show the experimental data and the line shows the fitted curve. The autocorrelation data for CAII in PBS buffer was fitted for a single component with an autocorrelation time ($\tau$) of 163 µsec. The autocorrelation data for CAII in perikaryon and dendrite was globally fitted for three components with decay times of 426 µsec, 2260 µsec and 11620 µsec. The concentrations of each component in the perikaryon and dendrite are shown in Fig. 6B, with standard deviations indicated. The difference in concentration between perikaryon and dendrite was significant for the 426 µs component ($p = 0.0034$) and the 11620 µs component ($p = 0.123$), according to the two-tailed Student t-test.

Fig 6. Transport metabolons generate pH microdomains

Cytoplasmic CAII reversibly converts intracellular CO$_2$ and H$_2$O into HCO$_3^-$ and H$^+$. CAII can form a transport metabolon with NHE to facilitate H$^+$ export, causing local H$^+$ depletion to generate alkaline pH microdomains. CAII can also form a transport metabolon with NBC to facilitate HCO$_3^-$ export, causing local accumulation of H$^+$ to generate acidic pH microdomains.
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6

\[ \text{extracellular} \quad \text{intracellular} \]

\[ \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^- \]

\[ \text{HCO}_3^- \quad \text{H}^+ \]

\[ \text{NHE} \quad \text{CAII} \]

\[ \text{high pH} \quad \text{low pH} \]

\[ \text{CAII} \]

\[ \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^- \]