Sodium-Bicarbonate Cotransport in Retinal Astrocytes and Müller Cells of the Rat

ERIC A. NEWMAN

Department of Physiology, University of Minnesota, Minneapolis, Minnesota

KEY WORDS glial cells; retina; pH; imaging; BCPCF; pH regulation

ABSTRACT Sodium-bicarbonate cotransport in retinal glial cells was studied in the everted eyecup preparation of the rat. Intracellular pH was monitored with the indicator dye BCPCF and fluorescence confocal microscopy. Raising the K\(^+\) concentration from 3 to 12 mM in HCO\(_3\)-buffered perfusate evoked an intracellular alkalinization in both astrocytes and Müller cells. The alkalinization developed more rapidly and was larger in astrocytes. The K\(^+\)-induced alkalinization was HCO\(_3\)-dependent; it was reduced by 33% in astrocytes and 71% in Müller cells when HCO\(_3\) was removed from the perfusate. The alkalinization was effectively blocked by addition of 0.5 mM 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS). Removal of Na\(^+\) from the perfusate evoked a rapid acidification in both types of glial cells. The results indicate that astrocytes and Müller cells in situ in the rat retina possess an electrogenic Na\(^+\)/HCO\(_3\)-cotransporter.


INTRODUCTION

Regulation of pH within the nervous system is essential for maintaining normal neuronal function. Ion channels (Barnes et al., 1993; Harsany and Mangel, 1993; Tombaugh and Somjen, 1996), neurotransmitter receptors (Traynelis and Cull-Candy, 1990; Tang et al., 1990; Vyklicky et al., 1990), and gap junctions (Spray and Bennett, 1985; Kettenmann et al., 1990) are all, to varying degrees, modulated by extracellular or intracellular pH, and variations in extracellular pH can lead to substantial changes in neuronal behavior (Balestrino and Somjen, 1988; Taira et al., 1993; Barnes et al., 1993; Gottfried and Chesler, 1994).

Neuronal activity causes characteristic changes in extracellular pH (pH\(_o\)) (Chesler and Kaila, 1992). In many brain regions, activity generates a transient alkalinization (reflecting synaptic activity), followed by a slower acidification (Chen and Chesler, 1992; Tong and Chesler, 1998). In the retina, alkaline shifts in pH\(_o\) predominate (Borgula et al., 1989; Yamamoto et al., 1992). Glial cell activity also contributes to variations in pH\(_o\) (Grichtchenko and Chesler, 1994a; Newman, 1996) and may serve to counterbalance the pH changes generated by neurons (Ransom, 1992). This pH regulatory flux is mediated by the electrogenic Na\(^+\)/HCO\(_3\)-cotransport system of glial cells.

The Na\(^+\)/HCO\(_3\)-cotransporter has been described in many glial cells, including astrocytes in brain slices (Grichtchenko and Chesler, 1994a; Grichtchenko and Chesler, 1994b), in the optic nerve (Aston and Orkand, 1988), and in culture (Boyarsky et al., 1993; O'Connor et al., 1994; Brune et al., 1994; Brooks and Turner, 1994), oligodendrocytes in culture (Kettenmann and Schlue, 1988; Boussouf et al., 1997), and in leech glial cells (Deitmer and Schlue, 1989). In the retina, the Na\(^+\)/HCO\(_3\)-cotransporter has been studied in dissociated amphibian (Newman, 1991; Newman, 1996) and elasmobranch (Newman, 1990) Müller cells.

The goal of the current study was two-fold. First, to confirm that the Na\(^+\)/HCO\(_3\)-cotransporter is present in glial cells in intact, acutely isolated tissue; second, to determine whether the cotransporter is present in mammalian retinal glial cells. Glial cells were studied in the intact rat retina, rather than in dissociated cell or

Grant sponsor: National Institutes of Health; Grant number: EY04077.

*Correspondence to: Dr. Eric A. Newman, 6–255 Millard Hall, Department of Physiology, University of Minnesota, 435 Delaware Street S.E., Minneapolis, MN 55455. E-mail: ean@tc.umn.edu

Received 6 January 1999; Accepted 2 February 1999

© 1999 Wiley-Liss, Inc.
culture preparations, as employed in earlier studies. The results indicate that both astrocytes and Müller cells of the intact mammalian retina possess a Na⁺/HCO₃⁻ cotransport system.

MATERIALS AND METHODS
Everted Eyecup Preparation

Male Long-Evans rats (300–400 gms) were killed with an overdose of sodium pentobarbital (200 mg/kg) injected intraperitoneally and the eyes removed. A small portion of the eye, cut from the back of the eyeball, was everted over a Plexiglas dome by lowering a sheet of Plexiglas with a hole (centered over the dome) cut into it. The Plexiglas sheet served to hold the eye in place, prevented the retina from detaching, and formed a seal preventing perfusate from leaking under the tissue.

The eyecup was incubated for 12 min at room temperature in collagenase/dispase (2 mg/ml) and DNase (0.1 mg/ml) in bicarbonate-buffered Ringer’s solution to digest the basal lamina at the inner surface of the retina and the vitreous humor, which were then removed by suction applied through a 28 gauge hypodermic needle. Following thorough rinsing in Ringer’s solution, the eyecup was incubated for 12 min in the pH indicator dye BCPCF-AM (50 µg/ml; Molecular Probes, Eugene, OR) and pluronic acid (1.75 mg/ml; Molecular Probes). Both astrocytes and Müller cells, but not retinal neurons, were well labeled with BCPCF. A similar labeling pattern had been observed previously with another membrane permeant dye (Newman and Zahns, 1998). In preliminary experiments, the isolated retina of the rat was used instead of the everted eyecup. Similar results were obtained using both preparations.

During experiments, the eyecup was perfused with oxygenated Ringer’s solution at 24°C. The preparation was viewed with a video rate confocal scanner (Noran Odyssey; Middleton, WI) and an upright microscope (Olympus BX60), with a 40X water immersion objective (0.8 NA).

Intracellular pH Measurements

Intracellular glial cell pH (pHi) was monitored with BCPCF, a derivative of BCECF modified to function as an emission ratio indicator dye (Liu et al., 1997). BCPCF was excited by the 488 nm argon laser line. Fluorescence emission was monitored at 500 nm (near the isosbestic emission wavelength) and at wavelengths longer than 515 nm. Images were acquired simultaneously at the two emission wavelengths. MetaMorph software (Universal Imaging, West Chester, PA) was used to capture and store images and to calculate ratio images.

Measurements of glial pHi in the intact retina proved more difficult to obtain than similar measurements made in freshly-isolated or cultured cells. Due to the inherent inefficiency of confocal imaging, the indicator dye bleached rapidly. Light exposure was kept to a minimum to prevent bleaching and cell damage, limiting the frequency of pHi measurements.

BCPCF Calibration

Intracellular pH measurements were calibrated using the nigericin-high K⁺ technique (Chaillet and Born, 1985). Calibration curves were obtained by perfusing eyecups in a series of HEPES-buffered nigericin solutions (pH 6.0 to 8.0). The resulting BCPCF ratios were fit by the equation (Newman, 1994),

\[
\frac{I_{515}}{I_{500}} = 1 + b \cdot \left( \frac{10^{(pH - pK_1)} - 10^{(7.0 - pK_1)}}{1 + 10^{(pH - pK_1)}} \right)
\]

where \(I_{515}/I_{500}\) represents the fluorescence ratio at the two emission wavelengths, normalized to the ratio at pH 7.0.

Reliable calibration curves were obtained for astrocytes imaged at the retinal surface, but not for Müller cells, which were imaged in the inner plexiform layer. It is likely that the calibration solutions could not penetrate the retina rapidly enough to permit Müller cell pHi to equilibrate with perfusate pH. (The lipophilic nature of nigericin presumably limited its diffusion into the retina.) For purposes of this study, BCPCF calibration in Müller cells was assumed to be the same as that in astrocytes. In astrocytes, the BCPCF fluorescence-pHi relation is described by equation (1) with \(b = 1.28 \pm 0.14\) and \(pK = 7.22 \pm 0.07\) (19).

Ideally, indicator dye calibration curves should be normalized by obtaining a nigericin calibration at a single pH value on each cell monitored during a study (Newman, 1994). It was not possible to obtain these single-point calibrations, however, as BCPCF bleaching during experiments was severe. Rather, single-point calibrations were run on a population of cells to obtain values for mean steady-state pHi. All other cells were then assumed to have a steady-state pHi equal to the mean value. Due to the near-linear BCPCF calibration relation close to steady-state pHi, small errors in steady-state pHi result in only minor errors in computed \(\Delta pHi\) values.

Whole-Cell Recording

The membrane potential of astrocytes and Müller cells was monitored with whole-cell patch-clamp recording. Everted eyecups were prepared using the same procedure employed for pH imaging except that BCPCF labeling was omitted and the preparation was incubated in collagenase/dispase and DNase for an additional 8 min following vitreous removal. Glial cells were identified by filling with Lucifer Yellow.
Solutions

Bicarbonate-buffered Ringer’s contained (in mM): NaCl, 117.0; KCl, 3.0; CaCl2, 2.0; MgSO4, 1.0; NaH2PO4, 0.5; dextrose, 15.0; NaHCO3, 26. It was equilibrated with 5% CO2 in O2 and had a pH of ~7.4 at 24°C. HEPES-buffered Ringer’s contained: NaCl, 135.0; KCl, 3.0; CaCl2, 2.0; MgSO4, 1.0; NaH2PO4, 0.5; dextrose, 15.0; HEPES, 10. It was adjusted to pH 7.4 with NaOH and equilibrated with 100% O2. In 12 mM K+ solutions, KCl was substituted for NaCl. In zero Na+ solutions, N-methyl-D-glucamine chloride was substituted for NaCl and choline bicarbonate for NaHCO3. The nigericin-high K+ calibration solution contained: N-methyl-D-glucamine, 18.5; KCl, 105.0; CaCl2, 2.0; MgSO4, 1.0; NaH2PO4, 0.5; dextrose, 15.0; HEPES, 30.0; nigericin, 20µM, and it was titrated with KOH. DIDS (Sigma, St. Louis, MO) was added to solutions immediately before use. The pipette solution for whole-cell recording contained: NaCl, 25; KCl, 112; CaCl2, 1; MgCl2, 7; Na2ATP, 5; EGTA, 5; HEPES, 1; Lucifer Yellow CH, 0.1%.

Statistics

Results are given as means ± S.D., with number of samples, n, in parentheses. The number of samples represents the number of experimental trials rather than the total number of cells. For each trial, measurements from 3 to 7 astrocytes and 75 to 200 Müller cells were averaged. Statistical significance was assessed using the Student’s t-test (unpaired samples).

RESULTS

Measurement of pHi in Retinal Glial Cells

Glial cells were identified by their morphology and by their location within the retina. Astrocytes were restricted largely to the nerve fiber layer at the vitreal surface of the retina and had multiple processes radiating from their somata, many of which contacted blood vessels (Fig. 1A). For pHi measurements, individual astrocyte somata were imaged. Müller cells were labeled throughout their length and could be followed from the vitreal surface to past their somata in the inner nuclear layer. For pHi measurements, Müller cell primary processes were imaged in the mid-inner plexiform layer, 20 to 30 µm beneath the retinal surface (Fig. 1C). Measurements were made from regions encompassing 75 to 200 Müller cell processes. In BCPCF calibration experiments, Müller cell processes surrounding neuronal somata in the ganglion cell layer were imaged (Fig. 1B). pH within Müller cells equilibrates rapidly and even when Na+/HCO3− cotransporters are local-
ized to a specific cellular region, pH$_i$ is essentially uniform throughout the cell (Newman, 1996). pH$_i$ measurements were made simultaneously from astrocytes and Müller cells in experimental trials by switching the plane of focus alternately between the retinal surface and the inner plexiform layer.

**Steady-State Intracellular pH**

Steady-state pH$_i$ was not stable in astrocytes and Müller cells, but rather drifted slowly in an acid direction over a number of hours (see Fig. 2). In general, the drift was more severe in Müller cells than it was in astrocytes. This drift contrasts with the stable steady-state pH$_i$ observed in dissociated Müller cells (Newman, 1996), and it may be due to the slow recovery of the retina following the trauma of enucleation and the cutting of the eyecup. Alternately, the drift may reflect the slow rundown of the preparation, perhaps due to lack of adequate perfusion.

Steady-state pH$_i$ was measured in retinas 1 to 1.5 h after perfusion of the preparation had commenced. pH$_i$ in astrocytes equaled 7.27 ± 0.13 (26). pH$_i$ in Müller cells, measured within the ganglion cell layer (6 to 8 µm beneath the surface) equaled 7.28 ± 0.06 (5). Müller cell pH$_i$ measured in the inner plexiform layer, equaled 7.51 ± 0.22 (5). The large variability in pH$_i$ measurements in the inner plexiform layer is most likely due to the difficulty of obtaining accurate nigericin calibrations deep within the retina. Müller cell pH$_i$ calibrations within the superficial ganglion cell layer were judged to be more accurate. For the purposes of calibration (see Methods), both astrocytes and Müller cells were assumed to have a steady-state pH$_i$ of 7.25. This value is somewhat higher than pH$_i$ measured in astrocytes in the brain (Chesler and Kraig, 1989; Grichtchenko and Chesler, 1994b).

**K$^+$-Induced Intracellular Alkalinization**

An alkalinization was evoked within both astrocytes and Müller cells when the K$^+$ concentration ([K$^+$]) in the perfusate was raised from 3 to 12 mM (Fig. 2). Raising [K$^+$] induced a rapid alkalinization in astrocytes, averaging 0.30 ± 0.07 (8) pH units, measured 12 min after switching to 12 mM K$^+$ solution. The alkalinization was normally complete within 5 min after switching solutions.

In contrast, the alkalinization evoked in Müller cells was substantially slower and smaller. Raising [K$^+$] to 12 mM induced an alkalinization averaging 0.17 ± 0.04 (8) pH units at 12 min. In every instance, the Müller cell alkalinization had not reached a steady-state at 12 min.

The difference in the time course of K$^+$-induced alkalinization in astrocytes and Müller cells could be due to an intrinsic difference in acid-base transport within these cells. Alternately, it could arise because of the longer time it takes for the high K$^+$ perfusate to reach the Müller cells within the retina.

![Fig. 2. K$^+$-induced alkalinization in retinal glial cells. Application of 12 mM K$^+$ in HCO$_3$-buffered perfusate evokes a rapid alkalinization in astrocytes and a slower, smaller alkalinization in Müller cells. The records also show the slow acid drift of pH, present in many preparations.](image)

Changes in cell membrane potential in response to raising perfusate [K$^+$] were monitored in order to distinguish between these two possibilities. In both astrocytes and Müller cells, raising [K$^+$] from 3 to 12 mM resulted in a rapid depolarization (Fig. 3). Although depolarization was somewhat slower in Müller cells, the depolarization was essentially complete within 1 min in both types of glial cells. Infiltration of the perfusate into the retina thus does not appear to limit the pH$_i$ response time of Müller cells. In mammalian Müller cells of species with vascularized retinas, including rat, most membrane K$^+$ conductance is localized near the soma (Newman, 1987). Thus, monitoring K$^+$-evoked depolarization serves as a good measure of K$^+$ diffusion into the retina.

**Bicarbonate Dependence of Intracellular Alkalinization**

The K$^+$-induced alkalinization in retinal glial cells could be generated by a depolarization-evoked activation of the Na$^+$/HCO$_3^-$ cotransport system, as it is in other glial cell preparations. This possibility was tested by determining the HCO$_3^-$ dependence and the stilbene sensitivity of the alkalinization.

The K$^+$-induced alkalinization was reduced, but not eliminated, when HCO$_3^-$ was removed from the perfusate (Fig. 4). In astrocytes, the alkalinization was reduced from 0.30 ± 0.07 (8) pH units in HCO$_3^-$-buffered perfusate to 0.20 ± 0.05 (5) pH units in HEPES-buffered perfusate (P < 0.01). Similarly, in Müller cells, the alkalinization was reduced from 0.17 ± 0.04 (8) to 0.05 ± 0.03 (5) pH units (P < 0.001). The K$^+$-induced alkalinization in HEPES was determined 1 to 1.5 h after switching from HCO$_3^-$ perfusate.

Substituting HEPES- for HCO$_3^-$-buffered perfusate produced a rapid glial cell alkalinization, due to the
reduction in pCO₂ in the perfusate, followed by a prolonged acidification. A steady-state pH was not reached for more than an hour after switching solutions, suggesting that it takes at least this long for the HCO₃⁻ in the retina to be washed out. The change in steady-state pH produced by switching from HCO₃⁻-buffered perfusate was large, averaging 0.47 ± 0.07 (3) pH units in astrocytes and 0.48 ± 0.06 (3) pH units in Müller cells, measured 12 min after removal of Na⁺. The acidifications are consistent with the presence of the Na⁺/HCO₃⁻ cotransporter, but may also be generated by the action of a Na⁺/H⁺ exchange system. Due to the rapid acidification produced by Na⁺ removal, it was not practical to measure K⁺-induced alkalinization in Na⁺-free perfusate.

**DISCUSSION**

**Na⁺/HCO₃⁻ Cotransport**

The results presented in this article indicate the presence of a Na⁺/HCO₃⁻ cotransport system in both
astrocytes and Müller cells in the intact rat retina. Raising extracellular \([K^+]\), which in turn depolarizes cells, induced an intracellular alkalinization in both types of glial cells. This alkalinization is generated, at least in part, by the \(Na^+/HCO_3^-\) cotransporter, as indicated by its \(HCO_3^-\)-dependence and DIDS sensitivity.

The \(K^+\)-induced alkalinization was only partially \(HCO_3^-\)-dependent. The \(HCO_3^-\)-independent component of the alkalinization could be generated by other acid/base transport systems or pumps (Pappas and Ransom, 1996). However, in other glial cell preparations, including salamander Müller cells (Newman, 1996), this component of the alkalinization is not generated by transmembrane acid/base flux and is most likely due to \(K^+\)-induced changes in the metabolic state of the cell (Orkand et al., 1973; Salem et al., 1975).

Even in the absence of \(HCO_3^-\) in the perfusate, it is likely that millimolar concentrations of \(HCO_3^-\) remain within the tissue. These levels are generated by the aerobic production of \(CO_2\), which is converted to \(HCO_3^-\). Thus, a component of the \(K^+\)-induced alkalinization observed in HEPES-buffered perfusate could be generated by \(HCO_3^-\)-dependent processes. It should also be noted that the total buffering capacity of glial cells is considerably reduced when HEPES-buffered perfusate is substituted for \(HCO_3^-\)-buffered perfusate (Chesler, 1990). Thus, observed alkalinizations in HEPES-buffered perfusate are generated by smaller acid/base fluxes than they are in \(HCO_3^-\)-buffered perfusate.

The \(K^+\)-induced alkalinization was effectively reduced by DIDS, a stilbene which blocks \(Na^+/HCO_3^-\) cotransport in some glial systems (Newman, 1991; O’Connor et al., 1994; Shrode and Putnam, 1994; Brune et al., 1994). Interestingly, the stilbenes DIDS and 4,4\'-dinitrostilbene-2,2\'-disulfonic acid (DNDS) are ineffective in blocking the cotransporter in gliotic brain astrocytes (Grichtchenko and Chesler, 1994a; Grichtchenko and Chesler, 1994b) and in cultured oligodendrocytes (Kettenmann and Schue, 1988). In Müller cells, addition of DIDS unmasked a \(K^+\)-induced acidification. The nature of this acidification is not known. It is not present in astrocytes of the rat retina, nor in dissociated salamander Müller cells (Newman, 1996).

The \(K^+\)-induced alkalinization was more rapid in time course and greater in magnitude in astrocytes than it was in Müller cells. This may indicate that cotransporter properties differ in the two types of glial cells. Alternately, these differences may reflect differences in transporter density or cell surface-to-volume ratios. The slow rise time of the alkalinization in Müller cells may also be due to the opposing \(K^+\)-induced acidification present in these cells.

The results suggest that, during steady-state conditions, the \(Na^+/HCO_3^-\) cotransporter generates a \(HCO_3^-\) influx into astrocytes and Müller cells in the rat retina. When cotransporter activity is reduced by HEPES substitution, the cells acidify, indicating an interruption of \(HCO_3^-\) influx. A similar acidification occurs when cotransporter activity is blocked by DIDS. It should be noted that the results are also consistent with \(HCO_3^-\) influx being generated by \(HCO_3^-\)-dependent and DIDS-sensitive transport processes other than the \(Na^+/HCO_3^-\) cotransporter. An influx of \(HCO_3^-\) through the cotransporter during steady-state conditions has been suggested in previous experiments (O’Connor et al., 1994; Newman, 1996).

**Function of \(Na^+/HCO_3^-\) Cotransport**

Retinal activation by light stimulation results in an extracellular alkalinization generated by increased synaptic activity, as well as by a reduction in the metabolic activity of photoreceptors (Borgula et al., 1989; Yamamoto et al., 1992). This change in \(pH_o\) can have profound effects on subsequent neuronal activity. Voltage-gated ion channels (Barnes et al., 1993; Harsanyi and Mangel, 1993; Tombaugh and Somjen, 1996), as well as neurotransmitter receptors (Traynelis and Cull-Candy, 1990; Tang et al., 1990; Vyklicky et al., 1990), are sensitive to \(pH_o\), and small variations in \(pH_o\) can lead to modulation of synaptic efficacy. In the salamander retina, for instance, an alkalinization of 0.05 pH units results in a 24% increase in synaptic transmission between photoreceptors and bipolar cells (Barnes et al., 1993).

The \(Na^+/HCO_3^-\) cotransporter of rat astrocytes and Müller cells may function to counter this alkalinization, thus helping to regulate retinal \(pH_o\) (Ransom, 1992). In several glial cell preparations, including dissociated salamander Müller cells (Newman, 1996) and hippocampal slices (Grichtchenko and Chesler, 1994a), activation of the cotransport system results in acid efflux and an acidification of extracellular space. The acidification may counterbalance the alkalinization generated directly by neuronal activity. This mechanism of glial...
regulation of pH\textsubscript{o} remains to be confirmed experimentally.

**ACKNOWLEDGMENTS**

The author thanks Paul Ceelen for his excellent technical assistance and Janice L. Gepner and Kathleen R. Zahs for their helpful comments on the manuscript.

**REFERENCES**


Boussouf A, Lambert RC, Gaillard S. 1997. Voltage-dependent Na\textsuperscript{+}/HCO\textsubscript{3}{-} cotransporter and Na\textsuperscript{+}/H\textsuperscript{+} exchanger are involved in intracellular pH regulation of cultured mature rat cerebellar oligodendrocytes. Glia 19:74–84.


