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

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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. *GLIA 26:302–308, 1999.*

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 (Astion and Orkand, 1988), and in culture (Boyarsky et al., 1993; O'Connor et al., 1994; Brune et al., 1994; Brookes 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

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culture preparations, as employed in earlier studies. The results indicate that both astrocytes and Müller cells of the intact mammalian retina possess a Na^+/HCO_3^- 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 upright microscope (Olympus BX60), with a 40X water immersion objective (0.8 NA).

Intracellular pH Measurements

Intracellular glial cell pH (pH_i) 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 pH_i 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 pH_i measurements.

BCPCF Calibration

Intracellular pH measurements were calibrated using the nigericin-high K^+ technique (Chaillet and Boron, 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),

$$\frac{I_{>515}}{I_{500}} = 1 + b \cdot \left(\frac{10^{(pH-pK)}}{1 + 10^{(pH-pK)}} - \frac{10^{(7.0-pK)}}{1 + 10^{(7.0-pK)}} \right) \quad (1)$$

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 pH_i 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-pH_i 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_i 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 pH_i. All other cells were then assumed to have a steady-state pH_i equal to the mean value. Due to the near-linear BCPCF calibration relation close to steady-state pH_i, small errors in steady-state pH_i result in only minor errors in computed ΔpH_i 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_i 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; $CaCl_2$, 2.0; $MgSO_4$, 1.0; NaH_2PO_4, 0.5; dextrose, 15.0; $NaHCO_3$, 26. It was equilibrated with 5% CO_2 in O_2 and had a pH of ~7.4 at 24°C. HEPES-buffered Ringer's contained: NaCl, 135.0; KCl, 3.0; $CaCl_2$, 2.0; $MgSO_4$, 1.0; NaH_2PO_4, 0.5; dextrose, 15.0; HEPES, 10. It was adjusted to pH 7.4 with NaOH and equilibrated with 100% O_2. 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 $NaHCO_3$. The nigericin-high K^+ calibration solution contained: N-methyl-d-glucamine, 18.5; KCl, 105.0; $CaCl_2$, 2.0; $MgSO_4$, 1.0; NaH_2PO_4, 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; $CaCl_2$, 1; $MgCl_2$, 7; Na_2ATP, 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 pH_i 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 pH_i 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 pH_i 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^+/HCO_3^- cotransporters are local-

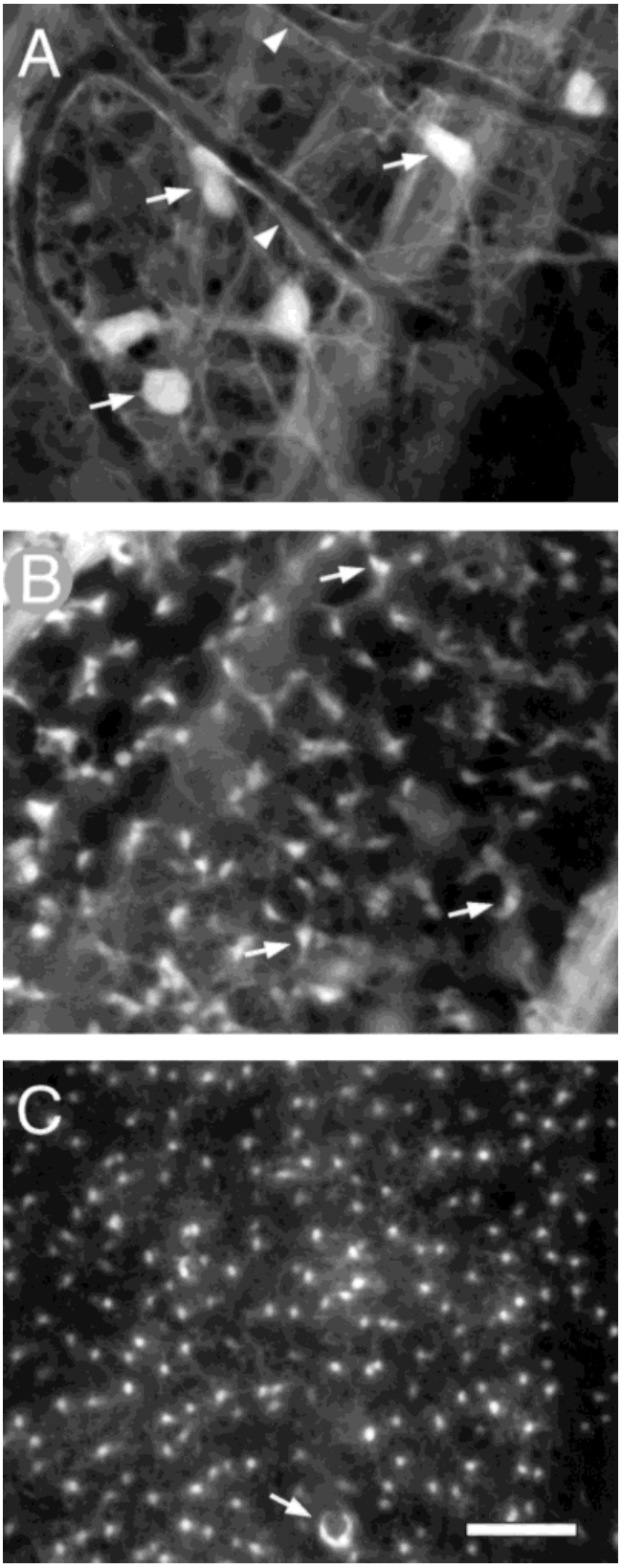

Fig. 1. Fluorescence confocal images of the everted rat eyecup labeled with the pH indicator dye BCPCF. **A:** Astrocytes (arrows) at the vitreal surface of the retina. Astrocyte processes terminating on blood vessels (arrowheads) are visible. **B:** Müller cell processes (arrows) surrounding the unlabeled somata of neurons in the ganglion cell layer. **C:** Müller cell processes (small, brightly labeled spots) in the inner plexiform layer. An astrocyte process surrounding a blood vessel (arrow) is also visible. Scale bar = 25 μm.

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 run-down 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.

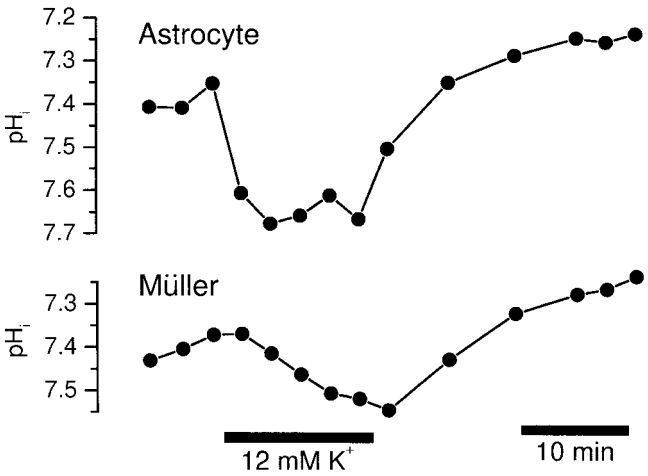

Fig. 2. K^+-induced alkalinization in retinal glial cells. Application of 12 mM K^+ in HCO_3^- perfusate evokes a rapid alkalinization in astrocytes and a slower, smaller alkalinization in Müller cells. The records also show the slow acid drift of pH_i present in many preparations.

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

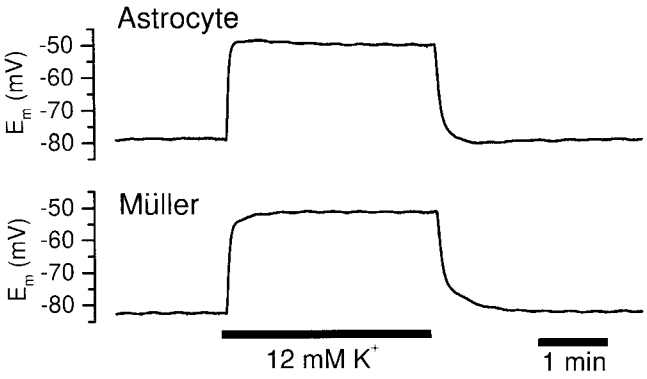

Fig. 3. K^+-induced depolarization in retinal glial cells. Application of 12 mM K^+ evokes a rapid increase in cell membrane potential (E_m) in both astrocytes and Müller cells. The two traces were recorded from the same preparation, but not simultaneously.

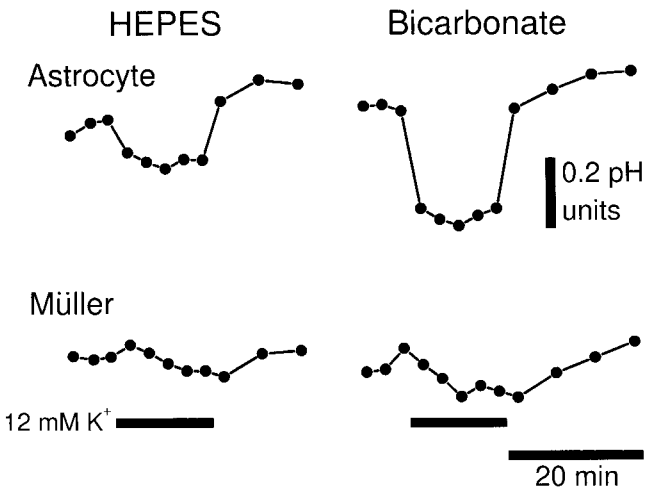

Fig. 4. Bicarbonate-dependence of K^+-induced alkalinization. pH_i records on the left were obtained in HEPES-buffered perfusate. The records on the right were obtained in the same preparation (but different cells) following substitution of HCO_3^--buffered perfusate. In both astrocytes and Müller cells, application of 12 mM K^+ induces a larger alkalinization in the presence of HCO_3^-.

reduction in pCO_2 in the perfusate, followed by a prolonged acidification. A steady-state pH_i was not reached for more than an hour after switching solutions, suggesting that it takes at least this long for the HCO_3^- in the retina to be washed out. The change in steady-state pH_i produced by switching from HCO_3^-- to HEPES-buffered perfusate could not be determined accurately due to the slow drift in pH_i in the preparation, although a net acidification beyond 7.25 was observed in all trials.

Effect of DIDS on Intracellular pH

If the K^+-induced alkalinization is generated by the Na^+/HCO_3^- cotransport system, it should be blocked by the stilbene 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS). This proved to be the case (Fig. 5).

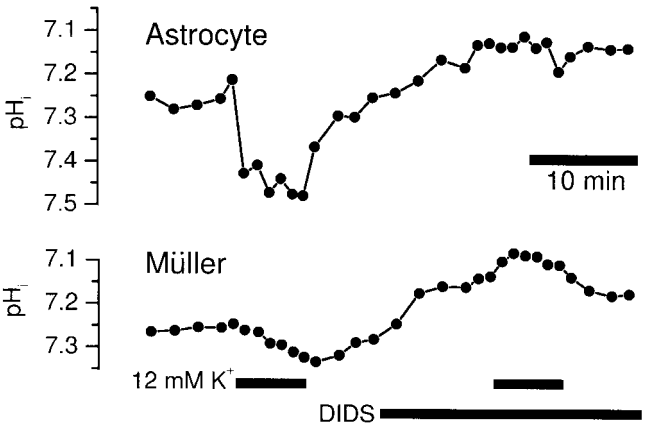

Fig. 5. DIDS sensitivity of K^+-induced alkalinization in HCO_3^- perfusate. In astrocytes, the K^+-induced alkalinization is blocked by addition of 0.5 mM DIDS. In Müller cells, the alkalinization is transformed into an acidification by DIDS. Addition of DIDS also produces an acid shift in steady-state pH_i in both astrocytes and Müller cells.

In astrocytes, addition of 0.5 mM DIDS to HCO_3^- perfusate reduced the K^+-induced alkalinization from 0.21 ± 0.05 (17) to 0.02 ± 0.04 (12) pH units ($P < 0.001$; measured at 12 min after addition of the high K^+ perfusate). In Müller cells, the pH_i response was actually transformed from a 0.09 ± 0.02 (13) pH unit alkalinization to a 0.07 ± 0.03 (8) pH unit acidification ($P < 0.001$). The K^+-induced acidification in the presence of DIDS was consistently observed.

Addition of 0.5 mM DIDS to the HCO_3^- perfusate also produced a rapid change in steady-state pH_i. In astrocytes, DIDS reduced steady-state pH_i 0.08 pH units to 7.17 ± 0.05 (17) ($P < 0.01$). In Müller cells, steady-state pH_i was reduced 0.11 pH units, to 7.14 ± 0.04 (13) ($P < 0.001$).

Effect of Na^+-Free Perfusate on Intracellular pH

Na^+/HCO_3^- cotransport activity should also be Na^+-dependent. Indeed, removal of Na^+ from the HCO_3^- perfusate generated a rapid acidification in both astrocytes and Müller cells (Fig. 6). The acidifications were 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^+/HCO_3^- 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^+/HCO_3^- Cotransport

The results presented in this article indicate the presence of a Na^+/HCO_3^- cotransport system in both

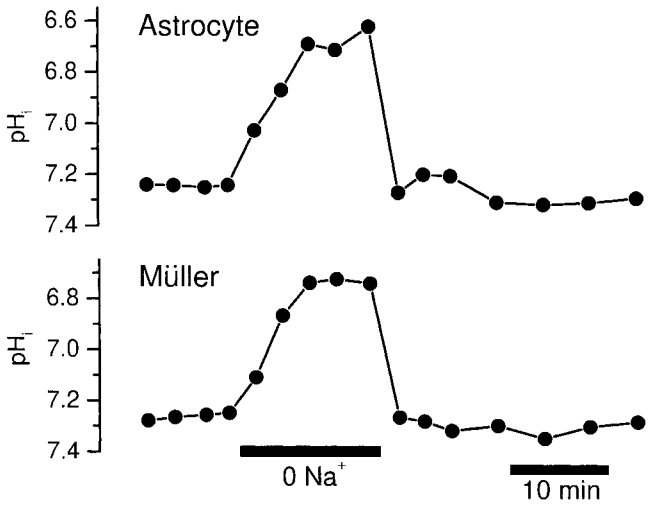

Fig. 6. Sodium dependence of glial cell pH_i. Substitution of nominally Na^+-free perfusate for control HCO_3^- perfusate evokes a large acidification in both astrocytes and Müller cells.

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, 1993). 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′-disulphonic acid (DNDS) are ineffective in blocking the cotransporter in gliotic brain astrocytes (Grinchtchenko and Chesler, 1994a; Grichtchenko and Chesler, 1994b) and in cultured oligodendrocytes (Kettenmann and Schlue, 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_o remains to be confirmed experimentally.

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