Intracellular pH Regulation in Cultured Astrocytes from Rat Hippocampus

I. Role of HCO_3^-

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ABSTRACT We studied the regulation of intracellular pH (pH_i) in single cultured astrocytes passaged once from the hippocampus of the rat, using the dye 2′,7′-biscarboxyethyl-5,6-carboxyfluorescein (BCECF) to monitor pH_i. Intrinsic buffering power (β_I) was 10.5 mM (pH unit)$^{-1}$ at pH_i 7.0, and decreased linearly with pH_i; the best-fit line to the data had a slope of −10.0 mM (pH unit)$^{-2}$. In the absence of HCO_3^-, pH_i recovery from an acid load was mediated predominantly by a Na-H exchanger because the recovery was inhibited 88% by amiloride and 79% by ethylisopropylamiloride (EIPA) at pH_i 6.05. The ethylisopropylamiloride-sensitive component of acid extrusion fell linearly with pH_i. Acid extrusion was inhibited 68% (pH_i 6.23) by substituting Li^+ for Na^+ in the bath solution. Switching from a CO_2/HCO_3^--free to a CO_2/HCO_3^--containing bath solution caused mean steady state pH_i to increase from 6.82 to 6.90, due to a Na^+-driven HCO_3^- transporter. The HCO_3^--induced pH_i increase was unaffected by amiloride, but was inhibited 75% (pH_i 6.85) by 400 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), and 65% (pH_i 6.55–6.75) by pretreating astrocytes for up to ~6.3 h with 400 μM 4-acetamide-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS). The CO_2/HCO_3^--induced pH_i increase was blocked when external Na^+ was replaced with *N*-methyl-D-glucammonium ($NMDG^+$). In the presence of HCO_3^-, the Na^+-driven HCO_3^- transporter contributed to the pH_i recovery from an acid load. For example, HCO_3^- shifted the plot of acid-extrusion rate vs. pH_i by 0.15–0.3 pH units in the alkaline direction. Also, with Na-H exchange inhibited by amiloride, HCO_3^- increased acid extrusion 3.8-fold (pH_i 6.20). When astrocytes were acid loaded in amiloride, with Li^+ as the major cation, HCO_3^- failed to elicit a substantial increase in pH_i. Thus, Li^+ does not appear to substitute well for Na^+ on the HCO_3^- transporter. We conclude that an amiloride-sensitive Na-H exchanger and a Na^+-driven HCO_3^- transporter are the predominant acid extruders in astrocytes.

KEY WORDS: H^+ concentration • acid–base transport • glia • nervous system • Na-H exchanger

INTRODUCTION

It is well established that the pH of the brain extracellular fluid (pH_{ECF})[1] can influence neuronal activity (for reviews, see Chesler and Kaila, 1992; Ransom, 1992), especially because many ion channels are sensitive to changes in extracellular pH (pH_o) (for reviews see Moody, 1984; Chesler, 1990). For example, the open probability of the *N*-methyl-D-aspartate-activated channel decreases at progressively lower pH_o values, with an apparent pK in the range 6.7–7.3 (Tang et al., 1990; Traynelis and Cull-Candy, 1990). The relationship between changes in pH_{ECF} and neuronal activity is complicated, however, because electrical activity itself can alter the pH of the brain extracellular fluid. The changes in pH_{ECF} elicited by neuronal firing are caused by the transport of acid–base equivalents across the plasma membrane of neurons and/or glia cells. This acid–base transport will have two effects. First, it obviously will affect the pH_i of the cells doing the transport, as well as the pH_{ECF} in the microenvironment. Second, because acid–base transporters on neighboring cells generally are sensitive to such pH_{ECF} changes, there will be indirect effects on the pH_i of these neighboring cells.

pH_{ECF} is thus in the position to mediate a complex interaction among neurons and glial cells. Indeed, the glial cells are thought to play a key role in regulating ECF composition, including pH_{ECF}, and the acid–base transporters of both invertebrate and mammalian glial cells are capable of modulating pH_{ECF} (see review by Deitmer and Rose, 1996). One such transporter thought to play an important role in regulating pH_{ECF} is the electrogenic Na/HCO_3 cotransporter. A similar transporter was first described in the cells of the proximal tu-

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[1]*Abbreviations used in this paper:* BCECF-AM, acetoxymethyl ester of the pH-sensitive dye 2′,7′-biscarboxyethyl-5,6-carboxyfluorescein; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; EIPA, ethylisopropylamiloride; $NMDG^+$, *N*-methyl-D-glucammonium; pH_{ECF}, pH of the brain extracellular fluid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

bule of salamander kidney (Boron and Boulpaep, 1983), where it moves Na^+ and HCO_3^- across the basolateral membrane from the cell to the blood, with a Na^+:HCO_3^- stoichiometry of 1:3 (Soleimani et al., 1987). The electrogenic Na/HCO_3 cotransporter is independent of Cl^-, but is inhibited by disulfonic stilbene derivatives, such as 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Electrogenic Na/HCO_3 cotransporters were identified subsequently in a number of glial cells, including giant neuropile glial cells (Deitmer and Schlue, 1987, 1989) and connective glial cells (Szatkowski and Schlue, 1992) of the leech, astrocytes in the optic nerve of *Necturus* (Astion and Orkand, 1988), and Müller cells from the salamander retina (Newman, 1991; Newman and Astion, 1991). In the Müller cells, the electrogenic Na/HCO_3 cotransporter has a Na^+:HCO_3^- stoichiometry of 1:3, and moves HCO_3^- out of the cells (Newman, 1991; Newman and Astion, 1991). On the other hand, in leech neuropile glial cells, and perhaps others as well, it is thought that the cotransporters have a Na^+:HCO_3^- stoichiometry of 1:2, and move HCO_3^- into the cells (Deitmer and Schlue, 1989; Deitmer, 1992; Munsch and Deitmer, 1994). It is not known whether the electrogenic Na/HCO_3 cotransporters with stoichiometries of 1:3 and 1:2 are different proteins.

There is also evidence that a Na/HCO_3 cotransporter might exist in mammalian glial cells. In primary cultures of astrocytes from the cerebral cortex of the mouse, the pH_i recovery from an acid load depends on Na^+ and HCO_3^-, is unaffected by acute Cl^- removal, but is inhibited by SITS (Chow et al., 1991). In astrocytes cultured from rat cerebellum, exposure to CO_2/HCO_3^- at 25°C causes a transient decrease in pH_i, followed by a sustained pH_i increase that has properties similar to those of the above mouse astrocytes (Brune et al., 1994). Both the mouse and rat data are consistent with mammalian astrocytes' having a Na/HCO_3 cotransporter that moves HCO_3^- into the cell and has a stoichiometry of either 1:2 (electrogenic) or 1:1 (electroneutral).

Two groups have used the whole-cell patch-clamp technique, one in rat astrocytes cultured from the cerebellum (Brune et al., 1994) and the other from the hippocampus (O'Connor et al., 1994), to address the question of whether Na/HCO_3 cotransporters in mammalian astrocytes are electrogenic. These authors recorded membrane voltage (V_m) and current (I_m) in astrocytes, while exposing them to CO_2/HCO_3^- at room temperature. They found that the exposure to CO_2/HCO_3^- elicited hyperpolarizations and outward currents that were at least partially inhibited by removing external Na^+ or applying DIDS, but were not inhibited by acutely removing external Cl^-. These data are consistent, but as discussed below, do not prove the hypothesis that introducing CO_2/HCO_3^- initiates the influx of HCO_3^- and negative charge via a DIDS-sensitive, electrogenic Na/HCO_3 cotransporter.

In this and the accompanying paper on cultured rat hippocampal astrocytes, we examine whether mammalian astrocytes do indeed possess an electrogenic Na/HCO_3 cotransporter. Our approach is significant in three ways. First, we perform the experiments at 37°C, where the activity of the electrogenic Na/HCO_3 cotransporter is likely to be greater than at room temperature. Second, we use a more specific assay for the cotransporter than monitoring V_m while adding CO_2/HCO_3^- under three conditions (control, DIDS, 0 Na^+). A potential difficulty with focusing on the DIDS-sensitive electrical changes elicited by exposing the cells to CO_2/HCO_3^- is that both DIDS and CO_2 are rather nonspecific amino-reactive agents. The isothiocyano groups on DIDS interact with amino groups via the Edman reaction. CO_2 interacts with susceptible free amines of proteins, resulting in the formation of carbamino compounds (Morrow et al., 1974), a classic example of which is the formation of carbamino hemoglobin. Moreover, the influx of CO_2 into the cell will rapidly lower pH_i, especially in the microenvironment on the inner surface of the membrane, and potentially change pH_i-sensitive ionic conductances. An additional problem is that switching to a HCO_3^--containing solution could evoke HCO_3^- currents per se. In cultured rat astrocytes, $GABA_A$-receptor channels can mediate HCO_3^- currents (Kaila et al., 1991).

Therefore, we have chosen to monitor pH_i and V_m while executing a maneuver (i.e., Na^+ removal) designed to elicit a response (i.e., a rapid depolarization) that is far more specific for the cotransporter than is the addition of CO_2/HCO_3^-. Although removing Na^+ per se is not specific, the resulting rapid depolarization can be attributed to only a few causes: inhibiting a Na^+-dependent K^+ conductance, inhibiting the Na-K pump, and forcing the electrogenic Na/HCO_3 cotransporter to move in the outward direction. Other Na^+-coupled transporters would either directly produce rapid V_m changes of the wrong sign, or indirectly produce slow V_m changes by changing cell composition. The Na^+-removal assay can be made even more specific by requiring that the rapid depolarization also depend on CO_2/HCO_3^- and be inhibited by DIDS.

Our third significant approach for identifying an electrogenic Na/HCO_3 cotransporter in hippocampal astrocytes is to determine whether the changes in V_m and pH_i are quantitatively consistent with electrogenic Na/HCO_3 cotransport. Thus, we compare the magnitude of the V_m change (from which we can compute the flux of charge) to the observed rate of pH_i change (from which we can compute the flux of HCO_3^-).

In the first of our two papers, we use a fluorescent pH-sensitive dye to evaluate the acid–base transporters responsible for regulating pH_i in both the presence and absence of CO_2/HCO_3^-. We found that the astrocytes have both a previously identified amiloride-sensitive Na-H exchanger, as well as a CO_2/HCO_3^--dependent transporter that requires external Na^+ and can be inhibited by the stilbene derivatives, DIDS and SITS. In the second paper (Bevensee et al., 1997), we provide evidence that the CO_2/HCO_3^--dependent transporter does not require Cl^-. This rules out a Na^+-driven Cl-HCO_3 exchanger. In addition, we used the perforated patch-clamp technique to record changes in V_m during the aforementioned Na^+-removal protocol, observing V_m changes nearly identical to those predicted from the rates of pH_i change. Our data indicate that hippocampal astrocytes have an electrogenic 1:2 Na/HCO_3 cotransporter.

METHODS

Solutions

The standard HEPES-buffered solution contained (mM): 125 NaCl, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4, 32 HEPES, and 10.5 glucose, titrated to pH 7.3 at 37°C with NaOH. 5% CO_2/17 mM HCO_3^--buffered solutions were made by substituting 17 mM $NaHCO_3$ for HEPES and adding 8.4 mM NaCl to maintain constant ionic strength. In NH_3/NH_4^+-containing solutions, NaCl was replaced with equimolar NH_4Cl. In 0-Na^+ solutions, the Na^+ substitute was either *N*-methyl-D-glucammonium ($NMDG^+$) or Li^+. In 0-Cl^- solutions, the Cl^- substitute was cyclamate, and total $[Ca^{2+}]$ was increased threefold to compensate for Ca^{2+} chelation by the anion substitute. In the 10-μM nigericin solution, KCl was 105 mM and the remainder of the Na^+ was replaced with $NMDG^+$. The acetoxymethyl ester of the pH-sensitive dye 2′,7′-biscarboxyethyl-5,6-carboxyfluorescein (BCECF-AM) was obtained from Molecular Probes, Inc. (Eugene, OR). Ethylisopropylamiloride (EIPA) was obtained from either E.J. Cragoe, Jr. (Nacogdoches, TX) or Research Biochemicals, Inc. (Natick, MA). DIDS was obtained from either Fluka Chemical Corp. (Ronkonkoma, NY) or Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from Sigma Chemical Co.

Cell Isolation and Culturing

Astrocytes were isolated and cultured from the hippocampi of 2–3-d-old rats using a modified version of the procedure described by McCarthy and de Vellis (1980). Rats were decapitated and the brains placed in chilled Dulbecco's PBS supplemented with 33 mM glucose. The hippocampi were removed from each brain by microdissection and placed in fresh, chilled PBS + 33 mM glucose. The hippocampi were triturated with pipettes of decreasing pore diameters until the solution was cloudy in appearance and contained only small hippocampal fragments. A final concentration of ~0.01% trypsin (GIBCO BRL, Life Technologies Inc., Gaithersburg, MD) was then added to the solution to digest the fragments further. After ~1 min, a final concentration of 25% fetal calf serum (Gemini Bioproducts, Inc., Calabasas, CA, or GIBCO BRL, Life Technologies Inc.) was added to the solution to inhibit further digestion by the trypsin. The solution was centrifuged (TJ-6; Beckman Instruments, Inc., Fullerton, CA) at 400 *g* for 5 min, and the cell pellet was resuspended in PBS + 33 mM glucose. This new suspension was again centrifuged and the pellet resuspended in the standard cell culture medium, which consisted of MEM supplemented with 28 mM glucose, 2 mM L-glutamine, 100 U ml^{-1} penicillin/streptomycin (GIBCO BRL, Life Technologies Inc.), and 10% fetal calf serum (Gemini Bioproducts, Inc. or GIBCO BRL, Life Technologies Inc.). After a final centrifugation, the pellet was resuspended in the culture medium and the cell suspension was plated into cell culture flasks. The cells were grown in a 5% CO_2, 37°C incubator, and the culture medium was changed every 3–5 d. To minimize the growth of oligodendrocytes in the cell culture, the flasks were agitated on a variable rotator (R4140; American Hospital Supply Corp., Miami, FL) at ~100 rpm for ~2 h before the me33dia was changed on the fourth day after cell plating. 10–12 d after cell plating, the flasks were again agitated at ~100 rpm for 18–24 h before the confluent monolayer of cells was treated with trypsin-EDTA (GIBCO BRL, Life Technologies Inc.) and passaged onto 22 × 22–mm glass coverslips. Before cell plating, the coverslips were washed with RadiacWash® (Biodex Medical Systems, Shirley, NY), and then 70–100% ethanol several times to rid the glass of grease and optimize cell attachment and growth. Experiments were performed on the cells 1–6 wk after the cells were passaged onto coverslips. The cells grown on the coverslips were immunocytochemically stained routinely with an antibody to the astrocyte-specific glial fibrillary acidic protein (GFAP). Over 95% of the cells stained positive for GFAP.

Measurement of pH_i in Single Astrocytes

Coverslips with attached astrocytes were first washed in a standard HEPES-buffered solution before being mounted in a perfusion chamber. The coverslip comprised the floor of the chamber. The chamber was then filled with a HEPES-buffered solution containing 10 μM BCECF-AM. Cells were incubated in this BCECF-AM solution, equilibrated with room air, for 10–20 min at 37°C. The perfusion chamber was then secured to the stage of a microscope equipped for epifluorescence (IM-35; Carl Zeiss, Inc., Thornwood, NY), and perfused for a minimum of 5 min with a HEPES-buffered solution to remove any unhydrolyzed BCECF-AM. Because the optical technique and the data acquisition used to measure pH_i have been described extensively elsewhere (Boyarsky et al., 1988), they will be only briefly summarized here. A 63× oil-immersion objective was used to choose a single astrocyte that was typically 20–40 μm in diameter, star shaped, and had a homogeneous distribution of dye inside the cytoplasm. pH_i recordings were obtained on cells that were nearly always surrounded by other cells. Dye was excited in an ~20-μm diameter area in the center of the cell; the light source was a 100-W halogen bulb. Approximately every 8 s, the dye was alternatively excited for 200 ms by light at wavelengths of 490 and 440 nm. The emitted fluorescence at a wavelength of 530 nm was amplified by a photomultiplier tube and recorded as I_{490} and I_{440}. Because I_{490} is pH dependent and I_{440} is relatively pH independent, the ratio of the two (I_{490}/I_{440}) is mainly a function of pH. Normalized I_{490}/I_{440} values were converted to pH_i using a high K^+/nigericin technique (Thomas et al., 1979), as modified for a single-point calibration by Boyarsky et al. (1988). Background I_{490} and I_{440} levels in the absence of dye were subtracted from the total I_{490} and I_{440} values.

In some experiments, pH_i was measured using a fluorescence imaging system in which the photomultiplier tube mounted on the microscope was replaced with an intensified CCD camera (ICCD-350F; Video Scope Int., Ltd., Sterling, VA). The system software included custom program macros written by Dr.

Michael Apkon (Department of Pediatrics, Yale University, New Haven, CT) for OPTIMAS (Optimas Co., Edmonds, WA).

In nine experiments on hippocampal astrocytes loaded with BCECF, applying 0.01% saponin caused the fluorescent signal to decrease to 4.3 ± 0.6% of the signal at the start of the experiment. Because 0.01% saponin is thought to permeabilize only the plasma membrane (Lin et al., 1990), we conclude that 96% of intracellular BCECF is located in the cytoplasm.

Intracellular Buffering Power

From experiments in which pH_i recovered from acute acid loads, we computed acid extrusion as the product of the rate of change in pH_i (dpH_i/dt) and total intracellular buffering power (β_T). Acid-extrusion rate (φ_E) thus has the units of moles per unit volume of cytoplasm per unit time (e.g., $\mu M\ s^{-1}$). In the subsequent manuscript (Bevensee et al., 1997), φ_L refers to acid loading and has similar units. β_T is the sum of the buffering power due to CO_2/HCO_3^- ($\beta_{HCO_3^-}$) and the buffering power due to intrinsic intracellular buffers (β_I). We computed the theoretical $\beta_{HCO_3^-}$ as $2.3 \times [HCO_3^-]$. As recently confirmed by our laboratory (Zhao et al., 1995), the actual $\beta_{HCO_3^-}$ is indistinguishable from the theoretical value. We calculated β_I in hippocampal astrocytes from the increases in pH_i elicited by increasing the external concentration of the weak base NH_3, as described by Roos and Boron (1981).

An example of an experiment for determining β_I in a single hippocampal astrocyte is shown in Fig. 1 *A*. We added 0.9 mM amiloride to all solutions to inhibit the Na-H exchanger previously identified in these cells (Pappas and Ransom, 1993), and removed the Cl^- to minimize potential Cl-base exchange (Vaughan-Jones, 1982). We acid loaded the astrocyte using the NH_4^+-prepulse technique (Boron and De Weer, 1976). Briefly, exposing the cell to a solution containing 20 mM NH_3/NH_4^+ caused pH_i to increase rapidly as NH_3 entered the cell and consumed H^+ to form NH_4^+ (Fig. 1 *A*, *ab*). In the continued presence of the NH_3/NH_4^+ solution, the pH_i decreased more slowly due to NH_4^+ influx and/or stimulation of acid-loading mechanisms (Fig. 1 *A*, *bc*). When the NH_3/NH_4^+ solution was removed, the pH_i decreased rapidly to a value lower than at the start of the experiment (Fig. 1 *A*, *cd*). As discussed below, pH_i normally recovers rapidly from such an acid load due to Na-H exchange. In Fig. 1 *A*, the pH_i recovery was greatly slowed by amiloride (Fig. 1 *A*, *de*). Exposing the cell sequentially to solutions containing 1, 2.5, and 5 mM NH_3/NH_4^+ caused pH_i to increase in a stepwise fashion (Fig. 1 *A*, *ef*, *gh*, and *ij*, respectively). However, especially at higher pH_i values, the rapid increase in pH_i in response to elevations of external NH_3/NH_4^+ was often followed by a slower decrease in pH_i (e.g., Fig. 1 *A*, *jk*). Therefore, we back extrapolated the pH_i vs. time record (see Fig. 1 *A*, *f'*, *h'*, and *j'*) to determine more accurately the pH_i changes due to the changes in NH_3/NH_4^+ (Aickin and Thomas, 1977). In Fig. 1 *B*, we plotted the results from five experiments similar to that shown in Fig. 1 *A*. β_I is plotted as a function of the average pH_i before and after a step change in NH_3/NH_4^+. β_I is 10.5 mM (pH unit)$^{-1}$ at pH_i 7.0. The best-fit line to the data has a slope of -10.0 mM (pH unit)$^{-2}$. Our β_I values are similar to those reported by Pappas and Ransom (1994), although they did not observe a dependence of β_I on pH_i.

Statistics

Data are reported as mean ± SEM. Means were compared using the paired and unpaired forms of the Student's *t* test (one-tail). $P < 0.05$ is considered significant. The pH_i dependence of β_I, as well as the SITS inhibition of the CO_2/HCO_3^--induced alkalinization were fitted by a straight line using a least-squares method.

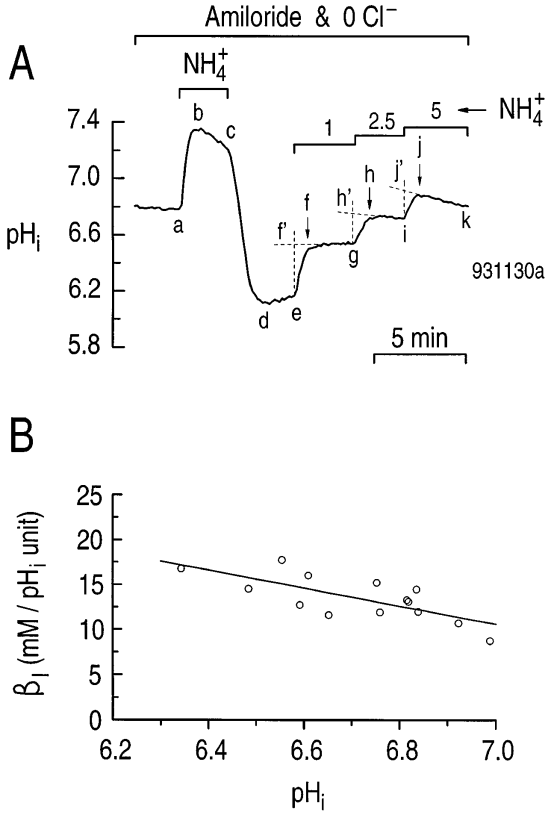

FIGURE 1. NH_3/NH_4^+ solutions can be used to calculate the pH_i dependence of intrinsic buffering power (β_I) in cultured astrocytes from the rat hippocampus. *(A)* The effect of step changes in $[NH_3/NH_4^+]$ on pH_i. A single astrocyte was bathed in a Cl^--free, HEPES-buffered solution containing 0.9 mM amiloride. After the cell was acid loaded by a brief exposure to 20 mM NH_3/NH_4^+ (*a–d*), the cell was subsequently exposed to solutions containing 1, 2.5, and 5 mM NH_3/NH_4^+. *(B)* pH_i dependence of intrinsic buffering power (β_I) in rat hippocampal astrocytes.

Rates of change in pH_i (dpH_i/dt) were fitted by either a third-order polynomial or a straight line using a least-squares method.

RESULTS

Initial Steady State pH_i in the Absence and Presence of CO_2/HCO_3^-

The astrocytes in this study, cultured from the rat hippocampus, had an average initial steady state pH_i of 6.83 ± 0.01 (n = 180) when exposed to a nominally CO_2/HCO_3^--free, HEPES-buffered solution (pH 7.3). The frequency distribution of these pH_i values is slightly skewed to more alkaline values (Fig. 2). For 63 of these 180 astrocytes, we switched the extracellular solution to one buffered with 5% CO_2/17 mM HCO_3^- (pH 7.3). In the presence of CO_2/HCO_3^-, these astrocytes had a mean steady state pH_i of 6.90 ± 0.01 (n = 63), 0.08 pH units higher, on average, than for the same cells in the absence of CO_2/HCO_3^- (6.82 ± 0.02). As indicated by the inset to Fig. 2, the frequency distribution of steady

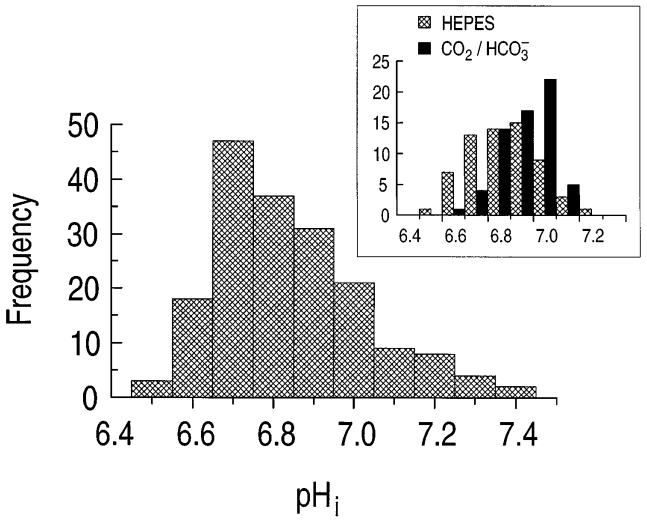

FIGURE 2. The distribution of steady state pH_i is bell-shaped for 180 hippocampal astrocytes bathed in a nominally CO_2/HCO_3^--free, HEPES-buffered solution. The bin width is 0.1 pH unit. *(inset)* The distribution of steady state pH_i of 63 astrocytes first exposed to HEPES is alkaline shifted when the cells are subsequently exposed to 5% CO_2/17 mM HCO_3^-.

state pH_i for the 63 astrocytes in the presence of CO_2/HCO_3^- (*solid bars*) is shifted toward more alkaline pH_i values, and has a smaller standard deviation than for cells in the absence of CO_2/HCO_3^- (*hatched bars*).

pH_i Recovery from an Acid Load in the Absence and Presence of CO_2/HCO_3^-

To study further the mechanisms by which astrocytes regulate pH_i, we acid loaded cells using the NH_4^+-prepulse technique (see METHODS). As shown in Fig. 3 *A*, when a single astrocyte in a HEPES-buffered solution was exposed to 20 mM NH_3/NH_4^+, pH_i increased rapidly (Fig. 3 *A*, *ab*), and then declined more slowly (Fig. 3 *A*, *bc*). When the astrocyte was returned to the standard HEPES-buffered solution, the pH_i sharply decreased (Fig. 3 *A*, *cd*), and then promptly recovered to a value similar to that at the start of the experiment (Fig. 3 *A*, *de*). A large component of the pH_i recovery of segment *de* was likely due to a Na-H exchanger previously identified in rat hippocampal astrocytes (Pappas and Ransom, 1993). Indeed, when the same astrocyte was acid loaded a second time (Fig. 3 *A*, *e–h*), the pH_i recovery was greatly slowed in the presence of 0.9 mM amiloride (Fig. 3 *A*, *hi*). The pH_i recovery remaining in the presence of amiloride may reflect incomplete block of the Na-H exchanger by amiloride, or activity of an H^+ pump (Pappas and Ransom, 1993). In cultured mouse astrocytes, Wuttke and Walz (1990) also observed an amiloride-insensitive, Na^+- and HCO_3^--independent pH_i recovery from an acid load, which may be due to acid efflux via lactate-H cotransport. Finally, switching the cell to a solution containing 5% CO_2/17 mM HCO_3^- led to a rapid pH_i increase (Fig. 3 *A*, *ij*), even though the Na-H exchanger was inhibited by amiloride.

From the segment-*de* pH_i recoveries in experiments similar to that shown in Fig. 3 *A*, we determined the pH_i dependence of total acid extrusion in HEPES ($\varphi_{HEPES} = dpH_i/dt \times \beta_T$; see METHODS) in the absence of amiloride and CO_2/HCO_3^- ("control" conditions).

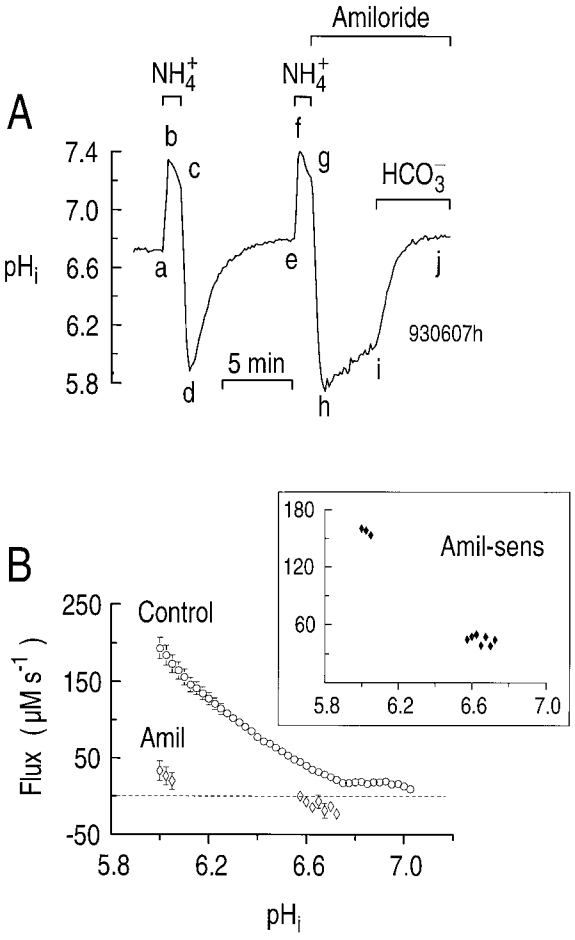

FIGURE 3. CO_2/HCO_3^- stimulates the pH_i recovery from an acid load when the Na-H exchanger is inhibited. (*A*) Effect of CO_2/HCO_3^- on the pH_i recovery from an acid load in the presence of amiloride. The single astrocyte was acid loaded twice by a brief exposure to a solution containing 20 mM NH_4Cl at a constant pH_O of 7.3 (*a–d* and *e–h*). During the indicated periods, 0.9 mM amiloride was present (*hj*), and 5% CO_2/17 mM HCO_3^- was present (*ij*). (*B*) The pH_i dependence of acid extrusion in a HEPES-buffered solution in the absence (*circles*) and presence (*open diamonds*) of 0.9 mM amiloride. In principle, the gap between the two groups of diamonds would have been filled had it been practical, in experiments such as that shown in *A*, to monitor the segment-*hi* pH_i recovery (which generated the diamonds near pH_i 6) until pH_i had reached the second group of diamonds (near pH_i 6.6). (*inset*) The pH_i dependence of the amiloride-sensitive acid extrusion (*closed diamonds*), obtained by subtracting the acid extrusion rate in the presence from that in the absence of amiloride.

The pH_i dependence of φ_{HEPES} is shown by the open circles in Fig. 3 *B*. We also determined the pH_i dependence of acid extrusion in the presence of amiloride (φ_{Amil}) from the segment *hi* pH_i increase observed in experiments similar to that shown in Fig. 3 *A*. These data are the group of three open diamonds near pH_i 6 in Fig. 3 *B*. We also computed φ_{Amil} from the pH_i decrease observed in other experiments in which we unmasked background acid loading by treating naive cells with 0.9 mM amiloride (data not shown). These data are the group of seven diamonds near pH_i 6.6 in Fig. 3 *B*.

At a pH_i of 6.05, φ_{HEPES} averaged 173 ± 12 μM s^{-1} ($n = 19$), whereas φ_{Amil} averaged only 20.1 ± 10.1 μM s^{-1} ($n = 4$). Therefore, at this pH_i, 88% of φ_{HEPES} was amiloride sensitive in the nominal absence of CO_2/HCO_3^-. The pH_i dependence of the amiloride-sensitive acid extrusion rate ($\varphi_{Amil\text{-}sens}$), defined as $\varphi_{HEPES} - \varphi_{Amil}$, is shown in the inset to Fig. 3 *B*.

In six experiments in which pH_i recovered in the presence of both amiloride and CO_2/HCO_3^- (e.g., Fig. 3 *A, ij*), acid extrusion increased 3.8-fold from 32.0 ± 12.9 μM s^{-1} (e.g., just before *i*) to 121 ± 29.2 μM s^{-1} (e.g., just after *i*) at a pH_i of 6.20 ± 0.05 ($P = 0.003$). The higher β_T in the presence of CO_2/HCO_3^- is factored into the above calculations. The data in Fig. 3 thus suggest that hippocampal astrocytes have as many as three acid-extrusion mechanisms: (*a*) an amiloride-sensitive Na-H exchanger (Fig. 3 *A*, *de* vs. *hi*); *(b)* possibly an amiloride-insensitive mechanism (e.g., H^+ pump) that functions in the nominal absence of CO_2/HCO_3^- (Fig. 3 *A*, *hi*); and *(c)* a CO_2/HCO_3^--dependent mechanism that can be observed even in the presence of amiloride (Fig. 3 *A*, *ij*).

If astrocytes possess both a Na-H exchanger and a HCO_3^- transporter, then the acid-extrusion rate should be greater in the presence than in the absence of CO_2/HCO_3^-. In the experiment shown in Fig. 4 *A*, we acid loaded an astrocyte twice in a HEPES-buffered solution, using NH_4^+ prepulses (Fig. 4 *A*, *a–d* and *e–h*). As we removed the NH_3/NH_4^+ the second time, we simultaneously introduced CO_2/HCO_3^-. Although the rates of pH_i change do not appear substantially different for the two pH_i recoveries, recall that β_T is substantially higher in the presence of CO_2/HCO_3^-. Moreover, it is clear from Fig. 4 *A* that pH_i recovered to a higher value in the presence vs. the absence of CO_2/HCO_3^- (compare points *i* and *e*). For six experiments similar to that shown in Fig. 4 *A*, we determined the pH_i dependence of acid extrusion both in the absence (φ_{HEPES}, *open circles*) and presence (φ_{HCO3}, *closed circles*) of CO_2/HCO_3^- (Fig. 4 *B*). Over a wide range of pH_i values, φ_E was as much as ~80 μM s^{-1} higher in the presence than in the absence of CO_2/HCO_3^-. Viewed differently, CO_2/HCO_3^- shifted the φ_E vs. pH_i curve 0.15–0.3 pH units in the alkaline direction.

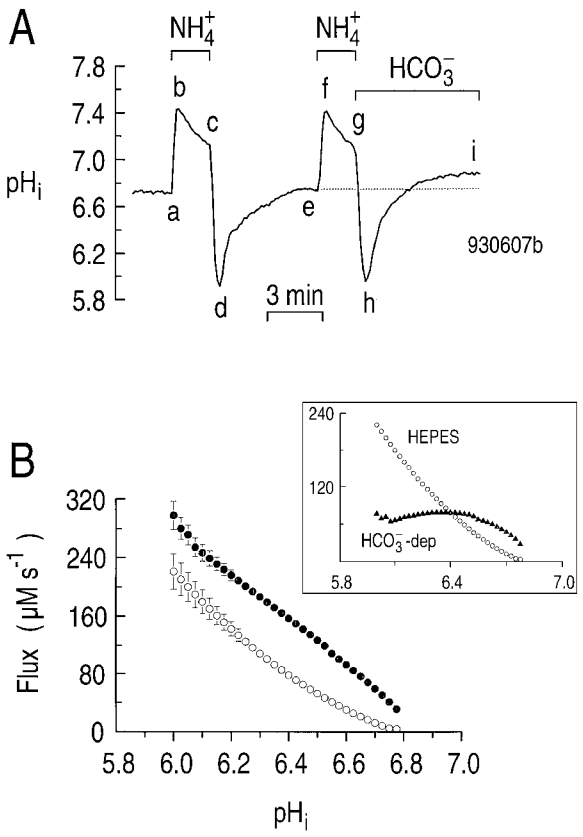

FIGURE 4. During the recovery from an acid load, acid extrusion is greater in the presence than in the absence of CO_2/HCO_3^-. *(A)* Effect of CO_2/HCO_3^- on the pH_i recovery from an acid load. An astrocyte was acid loaded twice by applying and withdrawing 20 mM NH_3/NH_4^+ (*a–d* and *e–h*). When NH_3/NH_4^+ was removed the second time, the cell was exposed to CO_2/HCO_3^- simultaneously (*g–i*). *(B)* The pH_i dependence of acid extrusion in the absence (*open circles*) and presence (*closed circles*) of CO_2/HCO_3^-. The data were taken from experiments similar to those in *A. (inset)* The open symbols are a replot of the data obtained in HEPES. The closed triangles represent the HCO_3^--dependent component of the flux, obtained by subtracting the open circles from the closed circles in the main panel.

In the inset to Fig. 4 *B*, we replot the φ_{HEPES} data from the main portion of Fig. 4 *B*, and also plot the difference between φ_{HCO3} and φ_{HEPES}, which is the HCO_3^--dependent flux ($\varphi_{HCO3-dep}$). As we saw in Fig. 3 *B*, φ_{HEPES} is the algebraic sum of HCO_3^--independent acid extrusion and acid loading mechanisms, and mainly reflects Na-H exchange. φ_{HEPES} is dominant at very low pH_i values. Therefore, astrocytes in the intact brain may predominantly use non-HCO_3^- transporters (e.g., Na-H exchange) to extrude acid under pathological conditions when pH_i is below ~6.4. Such low pH_i values have been recorded in astrocytes of rat in vivo during conditions of hyperglycemia and ischemia (Kraig and Chesler, 1990). $\varphi_{HCO3-dep}$ in Fig. 4 *B* is the algebraic sum of HCO_3^--dependent acid extrusion and acid load-

ing mechanisms, and thus could underestimate the HCO_3^- uptake mechanism. $\varphi_{HCO3-dep}$ is dominant in the "normal" pH_i range. Astrocytes in the intact brain may predominantly use HCO_3^- transporters to extrude acid when pH_i is above ~6.4 (i.e., in a more physiological pH range). Astrocytes in vivo typically have a steady state pH_i of ~7.0 (see review by Chesler, 1990).

Further Characteristics of Na-H Exchange

EIPA also inhibits the Na-H exchanger. Na-H exchange isoforms can be distinguished by their level of sensitivity to both amiloride and amiloride analogues such as EIPA. To characterize the pharmacology of the Na-H exchanger present in rat hippocampal astrocytes further, we examined how EIPA affects the pH_i dependence of acid extrusion in the nominal absence of CO_2/HCO_3^-. In the experiment represented in Fig. 5 *A*, we acid loaded an astrocyte by pulsing with 20 mM NH_3/NH_4^+ (Fig. 5 *A*, *a–d*), and then monitored the pH_i recovery with the cell exposed to 10 μM EIPA (Fig. 5 *A*, *de*). Using the segment-*de* pH_i recoveries from 13 similar EIPA experiments and 26 control experiments, we plotted the pH_i dependence of acid extrusion in the presence (φ_{EIPA}, Fig. 5 *B*, *open squares*) and absence (φ_{HEPES}, Fig. 5 *B*, *circles*) of EIPA. At a pH_i of 6.05, EIPA decreased φ_E from 173 ± 11.7 μM s^{-1} ($n = 19$) to 35.9 ± 25.7 μM s^{-1} ($n = 7$). Therefore, at a pH_i of 6.05, 79% of acid extrusion was inhibited by EIPA. In the four experiments discussed in Fig. 3 *B*, analyzed at this same pH_i, amiloride decreased φ_E to 20.1 ± 10.1 μM s^{-1} ($n = 4$), a reduction of 88%. Thus, it seems that amiloride is similar to EIPA in inhibiting Na-H exchange in cultured hippocampal astrocytes. The inset to Fig. 5 *B*, a plot of the EIPA-sensitive component of φ_E ($\varphi_{EIPA\text{-}sens}$), confirms that Na-H exchange activity is highest at low pH_i values and falls to nearly zero at a pH_i of ~6.8.

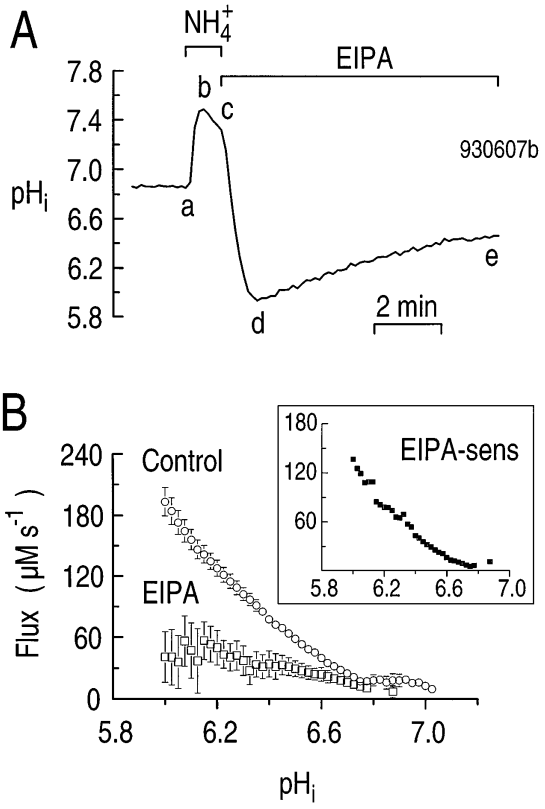

FIGURE 5. The pH_i recovery from an acid load in the nominal absence of CO_2/HCO_3^- is sensitive to EIPA. *(A)* Effect of EIPA on the pH_i recovery from an acid load. An astrocyte was acid loaded by applying and withdrawing 20 mM NH_3/NH_4^+ (*a–d*). EIPA (10 μM) was present during the pH_i recovery (*de*). *(B)* The pH_i dependence of acid extrusion in the absence (*circles*) and presence (*open squares*) of 10 μM EIPA. *(inset)* The pH_i dependence of the EIPA-sensitive component of acid extrusion (*closed squares*). These data were obtained by subtracting the open squares in the main panel from the open circles.

Na-H exchanger can apparently exchange Li^+ for H^+. Because Li^+ is a substrate for the Na-H exchanger at the brush border of renal proximal tubules (Aronson, 1985), we tested if Li^+ could substitute for Na^+ on the Na-H exchanger of the astrocyte. In the experiment shown in Fig. 6, we acid loaded a single astrocyte twice by pulsing with 20 mM NH_3/NH_4^+ (Fig. 6 *A*, *a–d* and *f–i*). During the pH_i recovery from the first acid load (Fig. 6 *A*, *de*), when Li^+ rather than Na^+ was the major cation, pH_i recovered more slowly than normal, and never reached the pH_i that prevailed at the start of the experiment (compare Fig. 6 *A*, *a* and *e*). When we returned the Na^+, pH_i increased further (Fig. 6 *A*, *ef*), possibly because the Na-H exchanger has a greater V_{max} for Na^+ than for Li^+ (Aronson, 1985). During the initial portion of the pH_i recovery from the second acid load (Fig. 6 *A*, *ij*), when Li^+ again was the major cation and the solution contained 0.9 mM amiloride, pH_i increased very slowly. Removing the amiloride (Fig. 6 *A*, *jk*) caused pH_i to increase nearly as fast as the recovery from the first acid load (Fig. 6 *A*, *de*). Returning the Na^+ caused the pH_i recovery rate to increase still further, and caused pH_i to return to its initial level (compare Fig. 6 *A*, *l* and *f*).

From segment-*de* data in five experiments, we determined the pH_i dependence of acid extrusion during the recovery of pH_i from an acid load when Li^+ rather than Na^+ was the major cation (Fig. 6 *B*, *open triangles*). For comparison, we also plot comparable control experiments performed on the same day (day-matched controls) in which Na^+ was the major cation (Fig. 6 *B*, *circles*). The pH_i dependence of the Li^+-dependent component of acid extrusion ($\varphi_{Li\text{-}dep}$) is shown in the inset to Fig. 6 *B*. At all pH_i values in the range studied, $\varphi_{Li\text{-}dep}$ in the presence of Li^+ was only 1/3 to 1/2 the value seen in the presence of Na^+. For example, at a pH_i of 6.23, acid extrusion was 125 ± 14 μM s^{-1} ($n = 5$)

with Na^+ as the dominant cation, but only 40.5 ± 5.4 μM s^{-1} (n = 5) with Li^+ as the dominant cation ($P < 0.0001$). At this same pH_i, amiloride (Fig. 6 *A*, *ij*) further reduced φ_E in the presence of Li^+ to 11.4 ± 1.8 μM s^{-1} (n = 5), an inhibition of 72% (P = 0.003). Therefore, amiloride-sensitive Li-H exchange appears to contribute to the pH_i recovery from an acid load when Li^+ rather than Na^+ is the major extracellular cation. Our data complement the finding that Li^+ can exchange for H^+ on the Na-H exchangers of both C6 and NN glial cell lines (Jean et al., 1986), as well as in cultured astrocytes from the cerebral cortex of the rat (Dixon and Wilson, 1995).

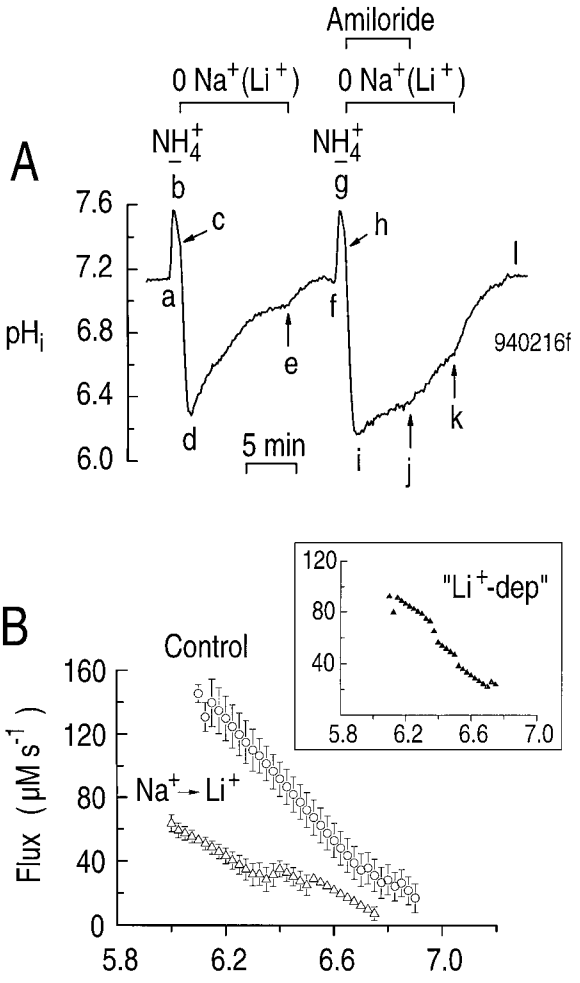

FIGURE 6. Li^+ can exchange with H^+ on the Na-H exchanger. *(A)* Effect of replacing Na^+ with Li^+ on the pH_i recovery from an acid load. An astrocyte was acid loaded twice by applying and withdrawing 20 mM NH_3/NH_4^+ (*a–d* and *f–i*). During the indicated times (*c–e* and *h–k*), Li^+ instead of Na^+ was the major extracellular cation. Amiloride (0.9 mM) was added immediately after the NH_3/NH_4^+ was removed (*h–j*). *(B)* The pH_i dependence of acid extrusion when Na^+ (*circles*) or Li^+ (*open triangles*) is the major cation. *(inset)*. The pH_i dependence of the "Li^+-dependent" acid extrusion (*closed triangles*). These data were obtained by subtracting the open triangles in the main panel from the open circles.

CO_2/HCO_3^--induced Alkalinization

CO_2/HCO_3^- elicits an increase in the mean steady state pH_i of astrocytes. Because the pH_i to which astrocytes recover after an acid load is higher in the presence than in the absence of CO_2/HCO_3^- (Fig. 4), one would predict that simply applying CO_2/HCO_3^- should increase steady state pH_i. As shown in the experiment in Fig. 7 *A*, when a single astrocyte was switched from a HEPES-buffered to a CO_2/HCO_3^--buffered solution, the pH_i transiently fell, due to the influx of CO_2 and production of intracellular H^+ and HCO_3^- (Fig. 7 *A*, *ab*), and then increased to a value higher than the initial one (Fig. 7 *A*, *bc*). When the astrocyte was returned to a HEPES-buffered solution, the opposite pH_i changes occurred (Fig. 7 *A*, *c–e*). The mechanism of the segment-*bc* pH_i increase is the subject of the rest of this paper, as well as the accompanying one (Bevensee et al., 1997). The mechanism of the segment-*de* decrease in Fig. 7 *A* is presently unknown. Some have suggested that a simi-

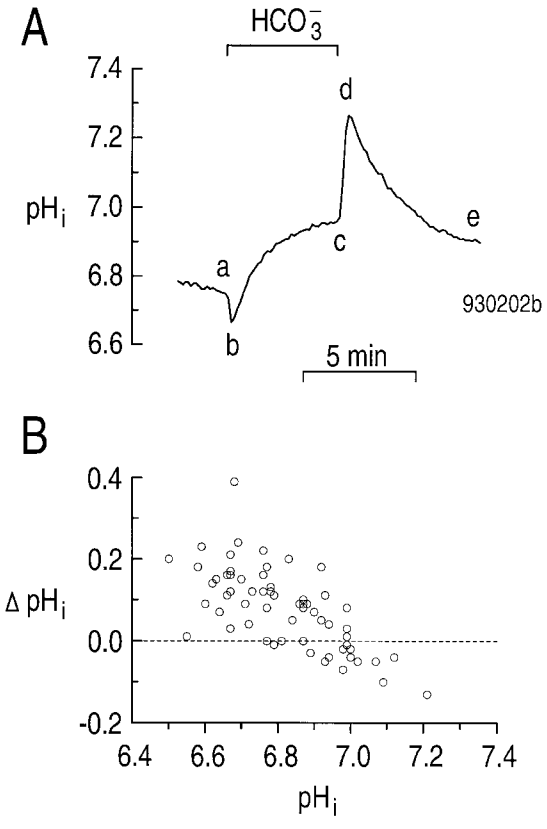

FIGURE 7. Mean steady state pH_i increases when hippocampal astrocytes are switched from HEPES to CO_2/HCO_3^-. *(A)* Effect of CO_2/HCO_3^- on steady state pH_i. During the indicated period, we switched to an extracellular solution that was buffered with 5% CO_2/17 mM HCO_3^- rather than HEPES (*a–c*). *(B)* The change in steady state pH_i caused by adding CO_2/HCO_3^-, plotted as a function of steady state pH_i in the HEPES-buffered solution.

lar pH_i decrease in other cells could be caused by Cl-HCO_3 exchange, with the HCO_3^- being provided by intracellular metabolism. However, as discussed in the accompanying paper (Bevensee et al., 1997), hippocampal astrocytes do not appear to have appreciable Cl-HCO_3 exchange activity. Another possibility is that the Fig. 7 *A*, *de* pH_i decrease is mediated by a K/HCO_3 cotransporter, similar to that described in squid axons (Hogan et al., 1995).

As we described in connection with the inset to Fig. 2, we performed 63 experiments similar to that shown in Fig. 7 *A*, in which we switched astrocytes from a HEPES-buffered to a CO_2/HCO_3^--buffered solution. The maximum φ_E at Fig. 7 *A*, *b* averaged 47.7 ± 2.4 μM s^{-1} at a pH_i of 6.74 ± 0.02 ($n = 63$). In Fig. 7 *B*, we plot the difference between the steady state pH_i in CO_2/HCO_3^- (Fig. 7 *A*, *c*) and the steady state pH_i in HEPES (Fig. 7 *A*, *a*) as a function of the initial steady state pH_i of the 63 astrocytes in HEPES (Fig. 7 *A*, *a*). Astrocytes with relatively low initial pH_i in HEPES are more likely to undergo a net increase in pH_i, whereas those with high initial pH_i are more likely to undergo a smaller increase in steady state pH_i when exposed to CO_2/HCO_3^-. In fact, for cells with an initial pH_i in the range of 6.9–7.2, CO_2/HCO_3^- actually caused the steady state pH_i to decrease in about half of the cells. This is why the distribution of steady state pH_i in CO_2/HCO_3^- (Fig. 2, *inset*) is narrower and alkaline shifted compared with the distribution in HEPES.

Amiloride does not inhibit the CO_2/HCO_3^--induced alkalinization. One possible explanation for the CO_2/HCO_3^--induced alkalinization in Fig. 7 *A*, *bc* is that CO_2/HCO_3^- stimulates Na-H exchange, as has been postulated for the proximal tubule (Chen and Boron, 1995*a*, 1995*b*). In six experiments in which the astrocytes had an average initial pH_i of 6.78 ± 0.06 in a HEPES-buffered solution, adding 0.9 mM amiloride caused pH_i to decrease to 6.66 ± 0.05 (data not shown), presumably because amiloride inhibited Na-H exchange that actively extruded acid in the steady state (see Fig. 3 *B, inset*). In contrast, adding 10 μM EIPA had no effect on the steady state pH_i ($n = 7$, data not shown). Introducing CO_2/HCO_3^- in the continued presence of amiloride elicited a rapid, initial decrease in pH_i, followed by a sustained increase (data not shown). In five experiments, the maximum acid extrusion rate was 89.3 ± 11.4 μM s^{-1} at a pH_i of 6.60 ± 0.03 during the CO_2/HCO_3^--induced alkalinization in the presence of amiloride. This average φ_E in the presence of amiloride is similar to φ_E in three day-matched control cells in the absence of amiloride (73.2 ± 4.8 μM s^{-1}; $P = 0.13$) at a similar pH_i of 6.67 ± 0.02. Therefore, even when Na-H exchange is substantially inhibited with amiloride, CO_2/HCO_3^- elicits an alkalinization in hippocampal astrocytes that have relatively low initial pH_i.

Stilbene derivatives inhibit the CO_2/HCO_3^--induced alkalinization. Because the most likely explanation for the CO_2/HCO_3^--induced alkalinization is stimulation of a HCO_3^--dependent acid extrusion mechanism, we tried inhibiting the alkalinization with stilbene derivatives that block HCO_3^- transporters in other preparations. Fig. 8 *A* shows an experiment on a single hippocampal

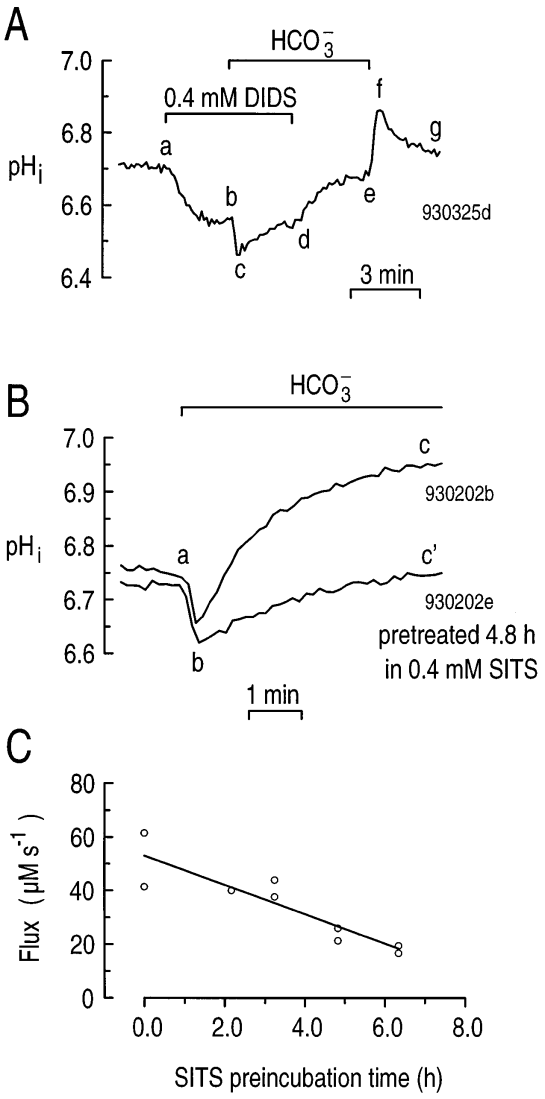

FIGURE 8. Stilbene derivatives partially inhibit the alkalinization when astrocytes are exposed to CO_2/HCO_3^-. (*A*) Inhibition by DIDS. At the indicated time, 400 μM DIDS was present in the extracellular solution (*a–d*). Between *b* and *e*, we switched the extracellular buffer from HEPES to 5% CO_2/17 mM HCO_3^-. (*B*) Inhibition by SITS. One of the two astrocytes was preincubated in 400 μM SITS for 4.8 h before the start of the experiment. Both cells were then exposed to CO_2/HCO_3^- (*a–c* and *a–c'*). *(C)* Maximum acid extrusion (i.e., at point *b* in *B*) as a function of the length of time astrocytes were preincubated in 400 μM SITS. All the experiments for this protocol were performed over a 2-d period. The best-fit line to the data has a slope of −5.5 μM s^{-1} h^{-1} and a *y*-intercept of 53.1 μM s^{-1}.

astrocyte exposed to CO_2/HCO_3^- in the presence of the stilbene derivative DIDS. Applying 400 μM DIDS in the nominal absence of CO_2/HCO_3^- elicited a sustained decrease in pH_i (Fig. 8 *A*, *ab*). The mechanism of this DIDS-stimulated acidification is unknown. In principle, some of the pH_i decrease could have been caused by inhibition of a HCO_3^--dependent acid extruder capable of using the small amount of HCO_3^- (~100 μM) present in the nominally CO_2/HCO_3^--free solution. However, it seems unlikely that such a mechanism, when inhibited, could produce the rapid segment-*ab* decrease in pH_i (Fig. 8 *A*). In the presence of DIDS, exposing the cell to CO_2/HCO_3^- caused a further acidification (Fig. 8 *A*, *bc*), followed by a slow recovery of pH_i (Fig. 8 *A*, *cd*) that is probably mediated by the Na-H exchanger. In the continued presence of CO_2/HCO_3^-, removing DIDS relieved most of the inhibition, causing pH_i to increase (Fig. 8 *A*, *de*). Removing the CO_2/HCO_3^- caused the usual series of pH_i changes (Fig. 8 *A*, *e–g*). Summarizing mean data, switching astrocytes from HEPES to CO_2/HCO_3^- in the presence of 400 μM DIDS resulted in a mean φ_E of 12.8 ± 3.2 μM s^{-1} at a pH_i of 6.79 ± 0.06 ($n = 7$). In day-matched control experiments in the absence of DIDS, φ_E was 51.0 ± 6.5 μM s^{-1} at a similar pH_i of 6.85 ± 0.06 ($n = 7$). Therefore, 400 μM DIDS inhibited the CO_2/HCO_3^--induced acid extrusion by 75% ($P < 0.0004$).

We also determined whether SITS, another commonly used stilbene derivative, can inhibit the CO_2/HCO_3^--induced alkalinization. Because 400 μM SITS has considerable fluorescence when excited at 490 and 440 nm, we exposed astrocytes to SITS in the culture media for up to ~6.3 h before experiments, and then washed away the drug.[2] In mesangial cells, the Na^+-driven Cl-HCO_3 exchanger is irreversibly inhibited when exposed to SITS for 1–2 h before the start of an experiment (Boyarsky et al., 1988). Fig. 8 *B* illustrates two day-matched experiments on single hippocampal astrocytes, one of which we pretreated with 400 μM SITS for ~4.8 h before the experiment. In the control cell, CO_2/HCO_3^- elicited an initial decrease in pH_i (Fig. 8 *B*, *ab*), followed by the expected increase (Fig. 8 *B*, *bc*). In the SITS-pretreated cell, the initial pH_i (before point *a*) was similar to that in the control cell, but the rate of pH_i increase (Fig. 8 *B*, *bc'*) was much slower. From experiments similar to those shown in Fig. 8 *B*, we plotted the maximum φ_E (pH_i 6.55–6.75) for nine astrocytes exposed to CO_2/HCO_3^- (point *b*) as a function of the time cells were pretreated with 400 μM SITS (Fig. 8 *C*). Compared with φ_E in astrocytes not treated with SITS (51.4 μM s^{-1}, $n = 2$), φ_E in cells treated with SITS for ~6.3 h (18.1 μM s^{-1}, $n = 2$) was inhibited 65%.

CO_2/HCO_3^--induced alkalinization requires external Na^+. Because several HCO_3^--dependent acid extruders also require Na^+, we performed experiments similar to those shown in Fig. 9 *A* to determine if Na^+ is required for the CO_2/HCO_3^--induced alkalinization in single hippocampal astrocytes. When we replaced external Na^+ with $NMDG^+$ in a HEPES-buffered solution, pH_i rapidly decreased (Fig. 9 *A*, *ab*), presumably due at least in part to inhibition and/or reversal of Na-H exchange. Reversal of Na-Ca exchange, by elevating intracellular Ca^{2+} and displacing H^+ from Ca^{2+} buffers, might also cause pH_i to decrease (Meech and Thomas, 1977). Switching to a CO_2/HCO_3^- buffer in the continued absence of external Na^+ caused pH_i to decrease further by ~0.06 (Fig. 9 *A*, *bc*). This pH_i decrease is

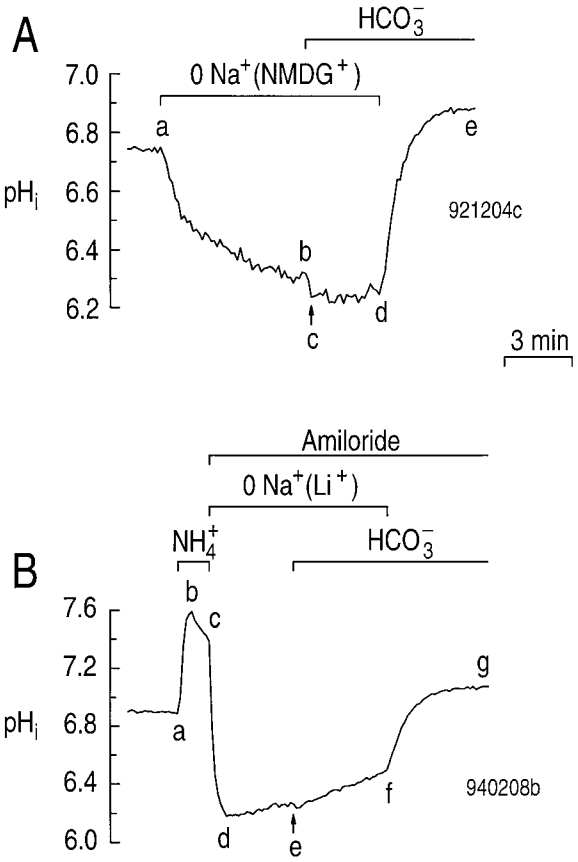

FIGURE 9. The HCO_3^- transporter is blocked when Na^+ is replaced with $NMDG^+$ or Li^+. *(A)* The effect of replacing Na^+ with $NMDG^+$ on the CO_2/HCO_3^--induced alkalinization. Between *a* and *d*, we replaced the extracellular Na^+ with $NMDG^+$. During *b–e*, we switched the extracellular solution to one buffered with 5% CO_2/17 mM HCO_3^-. *(B)* The effect of replacing Na^+ with Li^+ on the CO_2/HCO_3^--induced pH_i recovery from an acid load in the presence of amiloride. We exposed the astrocyte to 20 mM NH_3/NH_4^+ during *a–c*. Amiloride (0.9 mM) was present between *c* and *g*. Between *c* and *f*, Li^+ replaced extracellular Na^+. Finally, between *e* and *g*, the extracellular buffer was 5% CO_2/17 mM HCO_3^-.

[2]We noted that SITS-treated astrocytes assumed a more spherical shape, rather than the flat polygonal shape that characterized the other cells in this study.

small compared with Fig. 7, *ab* because the low pH_i in 0 Na^+ is closer to the pK of carbonic acid. However, there was no CO_2/HCO_3^--induced alkalinization (Fig. 9 *A*, *cd*). Switching the cell to a solution containing both Na^+ and CO_2/HCO_3^- elicited a rapid increase in pH_i to a value above that in the HEPES-buffered solution (compare Fig. 9 *A*, *e* and *a*). In five of six such experiments, removing extracellular Na^+ (Fig. 9 *A*, *ab*) caused pH_i to decrease from an average of 6.84 ± 0.08 to 6.50 ± 0.14. In one of the six experiments, the pH_i did not change appreciably when the cell was exposed to the Na^+-free solution. Exposing the six cells to CO_2/HCO_3^- in the continued absence of external Na^+ (Fig. 9 *A*, *bc*) caused a further decrease in the mean pH_i to 6.45 ± 0.11. The mean φ_E computed for Fig. 9 *A*, *cd* was only 4.0 ± 4.4 μM s^{-1}, compared with the maximum φ_E of 47.7 ± 2.4 μM s^{-1} at a pH_i of 6.74 ± 0.02 ($n = 63$) in experiments in which we added the CO_2/HCO_3^- in the presence of Na^+ (e.g., Fig. 7 *A*, *bc*). Because removing external Na^+ elicited a large decrease in pH_i, it was not possible to compare φ_E values in the presence and absence of Na^+ at the same pH_i. However, because other HCO_3^--dependent acid extruders are stimulated by low pH_i, we would have expected φ_E to be even larger at lower pH_i values prevailing in the presence of external Na^+. Therefore, the CO_2/HCO_3^--induced alkalinization requires external Na^+.

As discussed above, Li^+ can partially substitute for Na^+ on the Na-H exchanger in hippocampal astrocytes. To see if Li^+ could substitute for Na^+ on the transporter responsible for the CO_2/HCO_3^--induced alkalinization, we performed the experiment shown in Fig. 9 *B*. Our approach was to use an NH_4^+ prepulse to acid load an astrocyte in a CO_2/HCO_3^--free solution (Fig. 9 *B*, *a–d*), driving pH_i to a value similar to that prevailing in the absence of Na^+ (Fig. 9 *A*, *b*). With amiloride present and Li^+ as the dominant extracellular cation, pH_i recovered very slowly in the absence of CO_2/HCO_3^- (Fig. 9 *A*, *de*), presumably because the Na-H exchanger was almost completely blocked. Switching the cell to a solution buffered with CO_2/HCO_3^- increased the pH_i recovery rate only slightly (Fig. 9 *A*, *ef*). However, when we replaced the Li^+ with Na^+, pH_i rapidly increased (Fig. 9 *A*, *fg*) to a value higher than the pH_i at the start of the experiment (compare Fig. 9 *A*, *g* and *a*). In five experiments similar to that shown in Fig. 9 *B*, adding CO_2/HCO_3^- in the continued presence of amiloride and Li^+ increased φ_E only slightly, from 11.8 ± 1.6 μM s^{-1} (Fig. 9 *B*, *de*) to 20.0 ± 1.8 μM s^{-1} (Fig. 9 *B*, *ef*) at a pH_i of 6.23 ($P = 0.02$). On the other hand, returning Na^+ caused the mean φ_E to increase approximately sevenfold, to 145 ± 18 μM s^{-1} (Fig. 9 *B*, *fg*; $P < 0.001$). Therefore, Li^+ is a poor Na^+ substitute on the transporter responsible for the CO_2/HCO_3^--induced alkalinization in hippocampal astrocytes.

DISCUSSION

Properties of the Na-H Exchanger in Hippocampal Astrocytes

Na-H exchanger mediates the pH_i recovery from an acid load and contributes to the steady state pH_i. In this manuscript, we have investigated the major acid extruders responsible for regulating pH_i in astrocytes cultured from the hippocampus of the rat. Although we have focused predominantly on a HCO_3^--dependent acid extruder, we have also further characterized the Na-H exchanger that mediates almost the entire pH_i recovery from an acid load in the nominal absence of CO_2/HCO_3^- (Pappas and Ransom, 1993). We found that, at pH_i 6.05, amiloride inhibits 88% of acid extrusion during the pH_i recovery from an acid load. Moreover, our observation that applying amiloride leads to a decrease in steady state pH_i implies that the Na-H exchanger contributes to maintaining the steady state pH_i of the astrocytes, at least in the nominal absence of CO_2/HCO_3^-. In three experiments (not shown) on cells exposed to CO_2/HCO_3^-, we found that removing amiloride caused pH_i to increase by 0.03 ± 0.01, consistent with a modest role for the Na-H exchanger in maintaining steady state pH_i even in the presence of CO_2/HCO_3^-. Recently, Pizzonia et al. (1996) demonstrated that rat hippocampal astrocytes express the Na-H exchanger, NHE-1.

Although 10 μM EIPA inhibits the pH_i recovery from an acid load, it does not lower steady state pH_i. Because Na-H exchangers typically are sensitive to amiloride analogues such as EIPA, we studied the effect of EIPA both on the pH_i recovery from an acid load and on the steady state pH_i in hippocampal astrocytes. At very low pH_i values (i.e., 6.05), 10 μM EIPA inhibited the pH_i recovery from an acid load about as well as amiloride (79 vs. 88%). On the other hand, at progressively higher pH_i values, the amiloride-sensitive flux (Fig. 3 *B*, *inset*) fell towards zero more gradually than the EIPA-sensitive flux (Fig. 5 *B*, *inset*). Indeed, applying amiloride to a naive cell consistently elicited a pH_i decrease, presumably because it inhibited Na-H exchange and unmasked background acid loading, whereas applying EIPA did not. One explanation for these findings is that the astrocytes have two Na-H exchangers, one of which is less EIPA sensitive, particularly at high pH_i. Another explanation is that a single Na-H exchanger has a differential sensitivity to amiloride and EIPA that becomes especially apparent at high pH_i. A third possibility is that, at high pH_i, 10 μM EIPA simultaneously blocks Na-H exchange and alkalinizes the cell by an independent mechanism. Indeed, high levels of EIPA (i.e., 50 μM) elicit paradoxical alkalinizations in NIH-3T3 fibroblasts (Kaplan and Boron, 1994), rat osteoclasts (Ravesloot et al., 1995), and rat hippocampal CA1 neurons (Bevensee et al., 1996), as well as cultured astrocytes from the forebrain (Boyarsky et al., 1993;

Bevensee, M.O., G. Frey, and W.F. Boron, unpublished data) and hippocampus (Pizzonia et al., 1996).

Li^+ can substitute for Na^+ and exchange with H^+ on the Na-H exchanger. The astrocyte Na-H exchanger appears to exchange Na^+ for H^+ approximately fourfold faster than it exchanges Li^+ for H^+. In apical membranes of renal proximal tubules, the V_{max} for Li-H exchange is less than for Na-H exchange, although the affinity for Li-H exchange is higher (see review by Aronson, 1985). In NN and C6 glioma cells, the $K_{1/2}$ for Na^+ activation of the EIPA-sensitive $^{22}Na^+$ uptake was 17 and 50 mM, respectively. However, the $K_{1/2}$ for Li^+ inhibition of the EIPA-sensitive $^{22}Na^+$ uptake was only 5 and 9 mM, respectively (Jean et al., 1986).

Evidence for a Na^+-driven, HCO_3^--dependent Acid Extruder in Hippocampal Astrocytes

Five observations suggest that hippocampal astrocytes possess a Na^+-driven, HCO_3^--dependent acid extruder. (*a*) Acid extrusion during the recovery from an acid load is greater in the presence than in the absence of a CO_2/HCO_3^--buffered solution (Fig. 4). (*b*) When the pH_i recovery from an acid load is inhibited by amiloride in the nominal absence of CO_2/HCO_3^-, adding CO_2/HCO_3^- stimulates pH_i recovery (Fig. 3 *A*). (*c*) When astrocytes are switched from a solution buffered with HEPES to one buffered with CO_2/HCO_3^-, the average steady state pH_i increases (Fig. 7). (*d*) The CO_2/HCO_3^--induced alkalinization is inhibited by the HCO_3^--transport inhibitors DIDS (Fig. 8 *A*) and SITS (Fig. 8, *B* and *C*), but not by the Na-H exchange inhibitor amiloride. (*e*) The CO_2/HCO_3^--induced alkalinization requires external Na^+ (Fig. 9). These data are consistent with the Na^+-driven HCO_3^- transporter being either a Na^+-driven Cl-HCO_3 exchanger, or a Na/HCO_3 cotransporter. We address these possibilities in the subsequent manuscript (Bevensee et al., 1997).

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