Pflügers Arch Eur J Physiol (1994) 429:64–71

ORIGINAL ARTICLE

T. Brune · S. Fetzer · K.H. Backus · J.W. Deitmer

Evidence for electrogenic sodium-bicarbonate cotransport in cultured rat cerebellar astrocytes

Received: 8 March 1994 / Received after revision: 25 May 1994 / Accepted: 10 June 1994

Abstract We have studied the regulation of intracellular pH (pH_i), and HCO_3^--dependent membrane currents in cultured astrocytes from neonatal rat cerebellum, using the fluorescent pH-sensitive dye 2,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and the whole-cell patch-clamp technique. The steady-state pH_i was 6.96 in both nominally CO_2/HCO_3^--free, HEPES-buffered saline (6.96 ±0.14; n = 48) and in a saline containing 5% CO_2/24 mM HCO_3^- (6.96±0.18; n = 48) (at pH 7.4). Inhibition of the Na^+/H^+ exchange by amiloride (2 mM) caused a significant decrease of pH_i in nominally CO_2/HCO_3^--free saline. Addition of CO_2/HCO_3^- in the continuous presence of amiloride induced a large and fast intracellular alkalinization. Removal of external Na^+ also caused a fall of pH_i, and addition of CO_2/HCO_3^- in Na^+-free saline evoked a further fall of pH_i, while the outward current was reduced or even reversed. The stilbene 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS, 0.3 mM) reduced the pH_i recovery from the CO_2/HCO_3^--evoked acidification, and blocked the prominent intracellular acidification upon removal of CO_2/HCO_3^-. Removal of external Cl^- had little effect on these pH_i changes. Lowering the external pH from 7.4 to 6.6 in CO_2/HCO_3^--containing saline produced a large and rapid intracellular acidification and inward current, which were both greatly reduced by DIDS and in the absence of CO_2/HCO_3^-. The results suggest that the CO_2/HCO_3^--dependent current is partly due to a reversible bidirectional, electrogenic Na^+-HCO_3^- cotransporter, which helps to regulate pH_i in these cells. In addition, a prominent Na^+/H^+ exchanger contributes to extrude acid equivalents from these astrocytes to maintain the steady-state pH_i.

Key words Intracellular pH regulation · BCECF
Whole-cell patch-clamp · Rat astrocytes
Na^+-HCO_3^- cotransport · Na^+/H^+ exchange
Amiloride · DIDS

T. Brune · S. Fetzer · K.H. Backus · J.W. Deitmer (✉)
Abteilung für Allgemeine Zoologie, FB Biologie,
Universität Kaiserslautern,
Postfach 3049, D-67653 Kaiserslautern, Germany

Introduction

The pH regulation by glial cells plays an important role in the H^+ homeostasis in nervous systems. There is increasing evidence that glial cells not only regulate their intracellular pH (pH_i), but also help to regulate the pH in the extracellular, interstitial spaces between neurones and glial cells [7, 12, 27]. This may be mediated by changes in the glial membrane potential, as has been recorded in a variety of glial cells, where membrane depolarization, produced by changes in the external K^+ concentration, resulted in an intraglial alkalinization [9, 15]. Neuronal stimulation, which leads to a depolarization of glial cells, presumably due to accumulation of K^+ in the extracellular spaces (see [29]), also evokes an alkalinization in glial cells [8, 9, 28]. However, while the stimulation-induced and high-external K^+-evoked alkalinization in leech glial cells is dependent on the presence of CO_2/HCO_3^-, and sensitive to the stilbene 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), which blocks anion transport across cell membranes [15, 28], the K^+-induced alkalinization in rat cultured astrocytes appears to be present also in the nominal absence of CO_2/HCO_3^- [5, 25].

The existence of an electrogenic Na^+-HCO_3^- cotransporter may explain the dependence of glial pH_i regulation on the membrane potential [15]. An electrogenic Na^+-HCO_3^- cotransporter has been shown in giant leech glial cells [11, 13, 14], glial cells of the frog optic nerve [1], and Müller glial cells of the salamander retina [24], but not in rat cortical astrocytes [22]. Although there have been reports which suggest the presence of this cotransporter also in mammalian astrocytes [5, 10], there has been no direct and unambiguous evidence for electrogenic Na^+-HCO_3^- cotransport in these cells. A recent study [25] reports on a H^+ pump in rat hippocampal astrocytes, which may mediate the depolarization-induced intraglial alkalinization.

In the present study we have used the pH-sensitive fluorescent dye 2′,7′ bis (2-carboxyethyl)-5,6-carboxyfluorescein-acetoxymethylester (BCECF-AM) and whole-

cell patch-clamp recordings to measure changes in pH_i and membrane current induced by HCO_3^-. Our results suggest that the steady-state pH_i in rat cerebellar astrocytes is partly regulated by a Na^+/H^+ exchanger, and that a significant portion of the HCO_3^--dependent pH_i regulation is due to an electrogenic Na^+-HCO_3^- cotransporter.

Materials and methods

Cell culture

Primary cultures of enriched, glial fibrillary acidic protein-positive astrocytes (>95%) were obtained from cerebellar hemispheres of new born rats (P0–P1) as described by Fischer [17]. When the cell layer reached confluency, the cells were harboured and plated on glass cover slips coated with poly-D-lysine and kept at 7% CO_2/37°C. All experiments were done at room temperature (approx. 22–24°C) between 2 and 10 days after plating of the astrocytes.

Measurements of pH_i

Experiments on cultured astrocytes were performed with an inverted fluorescence microscope that was equipped with a Nikon CF-Fluor 10×objective (Diaphot, Nikon, Tokyo, Japan) and a dual excitation fluorometric imaging system (PTI, Wedel, Germany). The illumination was generated by a 75-W xenon bulb. Monochromator settings, chopper frequency and complete data aquisition were controlled by software for a microcomputer system (PTI). The BCECF fluorescence emission at the excitation wavelengths 440 and 495 nm of selected areas (diameter approx. 600 µm) of the astrocytic monolayer was recorded with a video camera using a 520-nm longpass filter (SIT C-2400, Hamamatsu, Garching, Germany), sampled at 3 Hz and computed into relative ratio units. pH_i and pH_i changes were determined by conversion of this ratio (440/495 nm) into pH units according to a calibration curve (Fig. 1). For calibration, cells were permeabilized with 10 µM nigericin to allow pH_i to approach the extracellular pH, buffered to values between 7.4 and 6.5 as described by Thomas et al. [30]. The ratio of the BCECF fluorescence was determined at four different pH values in extracellular solutions containing in mM: NaCl 10, KCl 135, $CaCl_2$ 1, $MgCl_2$ 1, glucose 24 and the following buffers: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 10 (pH 7.4), 3-(*N*-morpholino)propanesulphonic acid (MOPS) 10 (pH 7.1 and 6.8), or 1,4-piperazinediethanesulphonic acid (PIPES) 10 (pH 6.5), respectively (Fig. 1A). The means ratio values ± SD (n = 9) were plotted as a function of the pH to create the calibration curve (Fig. 1B).

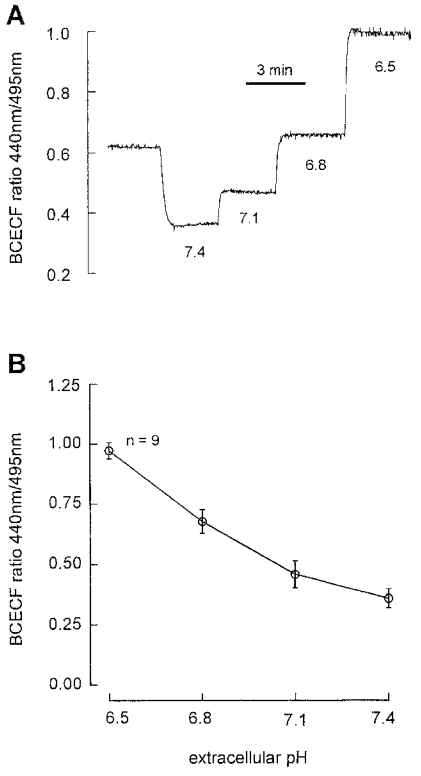


Fig. 1 **A** Calibration of the ratio signal at 440 nm/495 nm excitation wavelengths of intracellular BCECF (2′-7′-bis-carboxyethyl-5,6-carboxyfluorescein) in cultured astrocytes at different external pH in the presence of 10 µM nigericin. **B** The ratio signals of nine different BCECF-loaded cell cultures were plotted against the external pH, pH_o, mean ± SD

Electrophysiological procedures

For the electrophysiological experiments the culture dish was mounted on the stage of an inverted microscope. Recording pipettes were pulled from borosilicate glass, the tips were fire polished (resistances 2–4 MΩ). The pipette solution contained in mM: CsCl 140, NaCl 5, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10; pH adjusted to 7.2 with CsOH. The membrane currents were recorded using the whole-cell clamp configuration of the patch-clamp technique [16, 18]. We used $CaCl_2$ in the pipette to reduce the large background K^+ conductance and hence improve the space-clamp of the cell. The reference potential for all measurements was the zero-current potential of the pipette in the bath before establishment of the gigaseal. A 3 M KCl agar bridge was used as the reference electrode to reduce liquid junction potentials. In most experiments the series resistance was compensated by more than 80%. Currents were amplified by a List EPC-7 amplifier (Darmstadt, Germany), digitized, analysed, and stored with the aid of the software package TIDA (developed in the Institute of Neurobiology, Heidelberg, Germany) for a personal computer system.

Extracellular solutions

During the microfluorometric and electrophysiological experiments, the cell cultures in the recording chamber were continuously superfused with a standard solution (HEPES/standard, see Table 1). The effect of CO_2/HCO_3 was investigated by changing the HEPES-buffered standard solution to a CO_2/HCO_3^--buffered solution (Table 1). To investigate the dependence of the CO_2/HCO_3^--induced pH_i changes and currents on the extracellular pH and Na^+ concentration, the solutions listed in Table 1 were used. In another series of experiments the dependence on the extracellular Cl^- concentration was tested by changing the bath perfusion from a HEPES-buffered nominally Cl^--free solution (containing in mM: Na-gluconate 140, K-gluconate 5, Ca-gluconate 5, Mg-gluconate 1, glucose 24, HEPES 10; pH adjusted to 7.4 with NaOH) to a CO_2/HCO_3^--buffered Cl^--free solution (containing in mM: Na-gluconate; 116, $NaHCO_3$ 24, K-gluconate 5, Ca-gluconate 5, Mg-gluconate 1, glucose 24, HEPES 10; pH adjusted to 7.4 with NaOH). As similar experiments on glial cells in brain slices are being performed in a parallel study, the same solutions were used for all experiments, including the high glucose concentration.

Results

The steady-state pH_i of the cultured astrocytes, as obtained from measurements with BCECF, was 6.96±0.14

Table 1 Standard extracellular Na^+-free, and low pH, salt solutions as used in experiments (*NMDG N*-Methyl-D-glucamine, *HEPES* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, *PIPES* 1,4-piperazinediethinesulphonic acid)

Substance	Solution					
	HEPES standard	PIPES pH 6.6	CO_2/HCO_3^- standard	CO_2/HCO_3^- pH 6.6	HEPES Na^+ free	CO_2/HCO_3^- Na^+ free
NaCl	140	140	116	136.2	0	0
$NaHCO_3$	0	0	24	3.8	0	0
KCl	5	5	5	5	5	0
$CaCl_2$	1	1	1	1	1	1
$MgCl_2$	1	1	1	1	1	1
glucose	24	24	24	24	24	24
NMDG-HCL	0	0	0	0	140	140
$KHCO_3$	0	0	0	0	0	5
HEPES	10	0	10	0	10	10
PIPES	0	10	0	10	0	0
pH	7.4	6.6	7.4	6.6	7.4	7.4

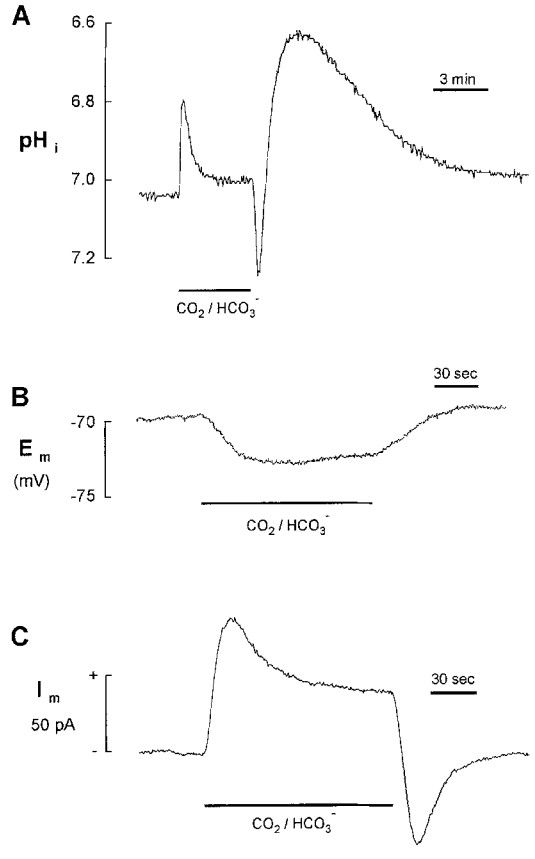


Fig. 2 The intracellular pH (pH_i) (**A**), membrane potential (E_m) (**B**) and whole-cell membrane current (I_m) (**C**) of cultured astrocyte(s) when CO_2/HCO_3^- was added to, and removed from, a HEPES-buffered saline; the pH of the saline was kept 7.4 throughout

(n = 48) in HEPES-buffered, nominally CO_2/HCO_3^--free saline, and 6.96±0.18 (n = 48) in saline containing in addition 5% CO_2/24 mM HCO_3^-; both salines buffered to 7.40±0.05.

The membrane resting potential, measured immediately after forming the whole-cell configuration was −64±3.7 mV (n = 24). The addition of CO_2/HCO_3^- to a HEPES-buffered saline resulted in a rapid intracellular acidification, from which the cells recovered (Fig. 2A), and a membrane hyperpolarization or outward current (Fig. 2B, C). The mean hyperpolarization of the cells measured in current-clamp mode was −2.3±0.3 mV (n = 7), and the outward current rose transiently to a maximum of 40.2±15.7 pA (n = 40), from where it often declined to a lower plateau.

Following the removal of CO_2/HCO_3^-, the pH_i transiently increased, presumably due to the exit of CO_2 from the cells, and then transiently fell well beyond its initial level in CO_2/HCO_3^--containing saline. Subsequently, the pH_i also recovered from this transient acidification and levelled near its initial steady-state (Fig. 2A).

The membrane hyperpolarization reversed within 30–90 s after removing CO_2/HCO_3 (Fig. 2B). The outward current transiently reversed to an inward current in most experiments upon removal of CO_2/HCO_3^-, before it returned to its original level (Fig. 2C).

Effects of amiloride

In order to dissociate different mechanisms contributing to the regulation of pH_i in these astrocytes, we used amiloride to inhibit the Na^+/H^+ exchanger present in the glial membrane [5, 10, 21]. Following the addition of amiloride, the cells acidified substantially (Fig. 3). Within 10–15 min the pH_i dropped by approximately 0.2–0.4 pH units.

In the continuous presence of amiloride, the addition of CO_2/HCO_3^- produced a large and rapid intracellular alkalinization (Fig. 3), on average, the pH_i increased by 0.18±0.08 pH units (n = 6). Removal of CO_2/HCO_3^- reversed this pH_i increase, and resulted in an even larger acidification, from which the cell could not recover. Only when amiloride was removed from the saline was there a fast pH_i recovery (Fig. 3).

It is concluded from this type of experiment first that, in the absence of CO_2/HCO_3^-, Na^+/H^+ exchanger maintains pH_i at an alkaline level against a considerable back-

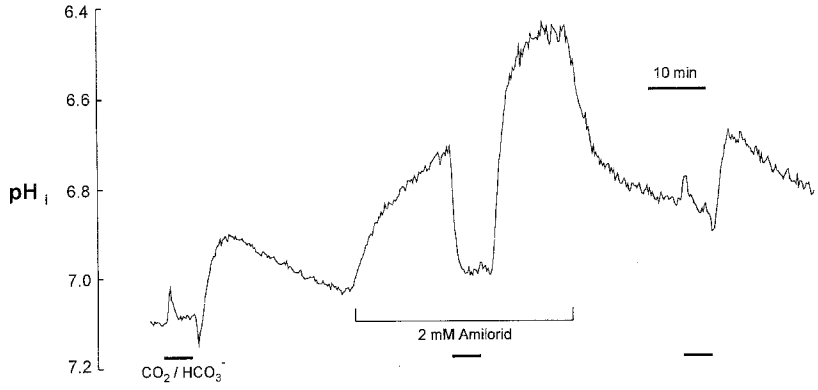


Fig. 3 The effect of amiloride (2 mM) on pH_i, and on the CO_2/ HCO_3^--induced changes of the astroglial pH_i

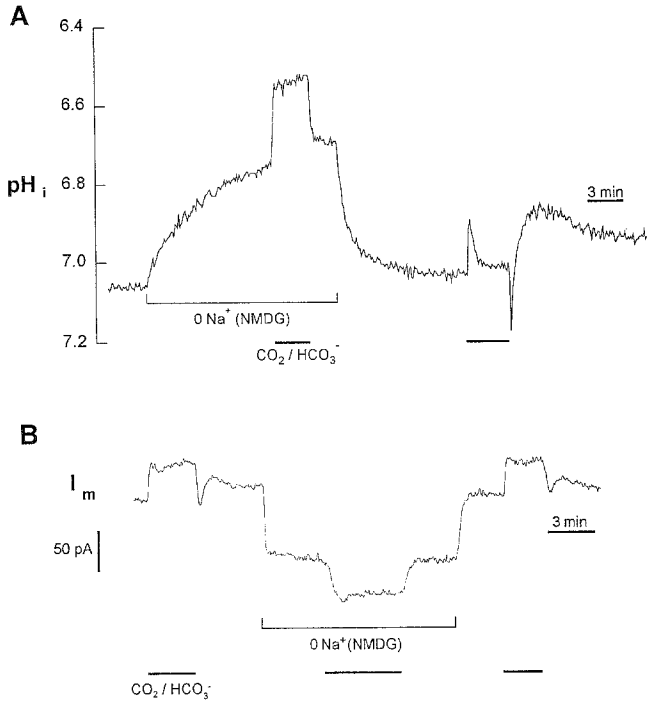


Fig. 4 The effect of the removal of external Na^+ (replaced by *N*-methyl-D-glucamine) on pH_i **(A)**, on membrane holding current **(B)**, and on the CO_2/HCO_3^--induced changes of pH_i **(A)** and membrane current **(B)**

ground acidification. Second, a large intracellular alkalinization upon the addition of CO_2/HCO_3^- was independent of amiloride-sensitive Na^+/H^+ exchange, indicating a HCO_3^--dependent mechanism of pH_i recovery from acidification. Third, upon removal of CO_2/HCO_3^- there was a large acidification, from which the cell did not recover, as long as amiloride was present. In all experiments of this kind, there was no pH_i recovery in an amiloride-containing, CO_2/HCO_3^-free saline, suggesting that Na^+/H^+ exchange is the only HCO_3^--independent pH_i-regulating mechanism in these cells.

The steady-state pH_i appeared to decrease continuously during the experiment shown in Fig. 3. To some extent this on-going increase in the BCECF 440 nm/495 nm ratio occurred in almost 50% of our experiments. It may indicate a true acidification of the cells; however, if the dye would slowly penetrate more acidic cell organelles, this would also produce an apparent pH_i decrease. The increase in the noise of the pH_i recording indicates that during this experiment dye bleaching and/or dye loss from the cells was relatively prominent. This may also contribute to some extent to the continuous change in the BCECF ratio.

pH_i and membrane current in Na^+-free saline

A similar protocol was repeated in the absence of external Na^+ (instead of amiloride), to block not only Na^+/H^+ exchange, but all Na^+-dependent pH_i regulation (Fig. 4).

Removal of external Na^+ in a HEPES-buffered, nominally CO_2/HCO_3^--free saline caused a considerable intracellular acidification (Fig. 4A), which was even faster and larger than that observed in amiloride. The pH_i fell by 0.3–0.6 pH units to 6.2–6.8 within 5–10 min in the absence of external Na^+, and approached a steady-state where H^+ is expected to be near its electrochemical equilibrium across the cell membrane (at a membrane potential of −65 mV and an external pH of 7.4, H^+ would be in equilibrium at a pH_i of 6.3).

Addition of CO_2/HCO_3^- to this Na^+-free saline evoked an intracellular acidification on average by 0.24±0.1 pH units (n = 5), from which the cells could not recover (Fig. 4A). The pH_i sometimes fell below a value of 6.0. Upon removal of CO_2/HCO_3^- this acidification was partially reversed, presumably due to exit of CO_2 from these cells. When external Na^+ was readded, there was a rapid return of the pH_i back to its initial alkaline value.

The membrane current induced by CO_2/HCO_3^-, which was outward in the presence of external Na^+, was reduced from 40.2 nA to 17±10 pA (n = 9) or even reversed to an inward current of −46.6±4.7 pA (n = 3) in Na^+-free saline (Fig. 4B). It should be noted that intracellular Na^+ could not decline to zero under these conditions, because the patch electrode contained 5 mM Na^+ (see Materials and methods). After readdition of external Na^+, a similar outward current could again be recorded upon CO_2/HCO_3^- addition as in the control (Fig. 4B).

Removal of external Na^+ alone evoked a large, reversible inward current [23], which was not studied any fur-

ther here. A small transient outward current following CO_2/HCO_3 removal was sometimes observed (Fig. 4B); its nature remains unknown.

The experiments indicate that the CO_2/HCO_3^--induced intracellular alkalinzation in normal saline, and at least some of the HCO_3^--induced membrane outward current, were dependent upon external Na^+. It is concluded therefore that the recovery from the CO_2/HCO_3^--induced intracellular acidification was partly, and the intracellular acidification following the removal of CO_2/HCO_3^- was predominantly, due to electrogenic Na^+-HCO_3^- cotransport, operating in the inward and in the outward direction, respectively. The outward current in normal saline and the reduced outward or inward current in Na^+-free saline upon addition of CO_2/HCO_3^- would be consistent with an inwardly and outwardly directed, electrogenic Na^+-HCO_3^- cotransporter, carrying more HCO_3^- than Na^+.

Effects of DIDS and Cl^--free saline on the CO_2/HCO_3^--induced pH_i changes and membrane currents

The stilbene DIDS is an inhibitor of anion transport through cell membranes, and has been applied to inhibit

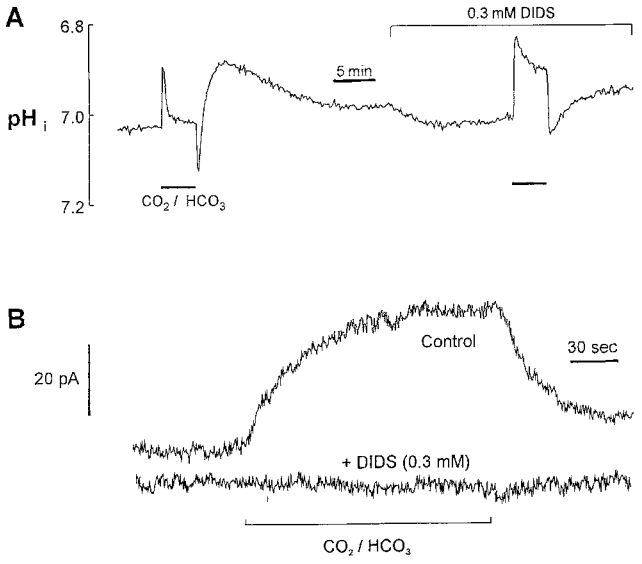


Fig. 5 The effect of the stilbene 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS, 0.3 mM) on the CO_2/HCO_3^--induced pH_i changes (**A**), and membrane outward current (**B**)

HCO_3^--dependent carriers [6]. The addition of DIDS (0.3 mM) to the external saline produced a small intracellular alkalinization (<0.05 pH units) in five out of seven cells (Fig. 5A). The pH_i changes and membrane current induced by CO_2/HCO_3^- were significantly altered in the presence of DIDS. The addition of CO_2/HCO_3^- induced a larger initial acidification than in the control and the subsequent pH_i recovery was considerably slowed (Fig. 5A). The pH_i levelled at a slightly lower value, on average at 6.93±0.06 pH units (n = 7), and both alkalinization and acidification upon removal of CO_2/HCO_3^- were greatly reduced by DIDS. The CO_2/HCO_3^--induced membrane current was greatly inhibited by DIDS (Fig. 5B); it was reduced to 5 pA on average, which was 8.6±4% (n = 7) of the control current without DIDS. The current recovered only a little after wash-out of DIDS and amounted to 18% of the control value, indicating that DIDS acted nearly irreversibly over the time period of our whole-cell clamp experiments (usually ≤40 min).

The removal of external Cl^- led to a slow intracellular alkalinization, which might be due to the inhibition of some residual Cl^-/HCO_3^- exchange, even in the nominal absence of CO_2/HCO_3^- (Fig. 6). The addition and removal of CO_2/HCO_3^- produced similar changes of pH_i in Cl^--free saline, and the steady-state pH_i levelled at a similar value as in the control, on average at 7.03±0.04 pH units (n = 6).

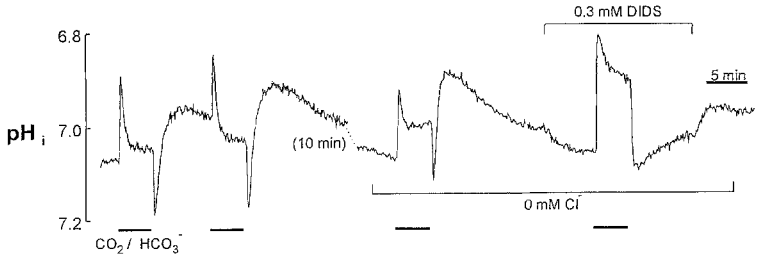
In the Cl^--free saline, DIDS exerted a very similar effect on the CO_2/HCO_3^--induced pH_i changes as in the presence of external Cl^- (Fig. 6); the initial CO_2/HCO_3^--induced acidification was sometimes even larger than in the control.

The experiments suggest that Cl^- plays a minor role in shaping the CO_2/HCO_3^--induced pH_i transients, while DIDS greatly affects them. This supports the identification of a Cl^--independent HCO_3^- carrier in the glial membrane, which is responsible for some of the recovery from the CO_2-induced intracellular acidification, and for the transient pH_i shift following the removal of CO_2/HCO_3^-.

pH_i and membrane currents at low external pH

Lowering the external pH from 7.4 to 6.6 induced a reversible decrease of pH_i, which was much faster and larger in the presence than in the absence of CO_2/HCO_3^-

Fig. 6 Exposure to CO_2/HCO_3^- in normal saline and after removal of external Cl^- and the effect of DIDS in the absence of external Cl^-. Note that removal of external Cl^- itself has only a small effect on the CO_2/HCO_3^--induced pH_i changes



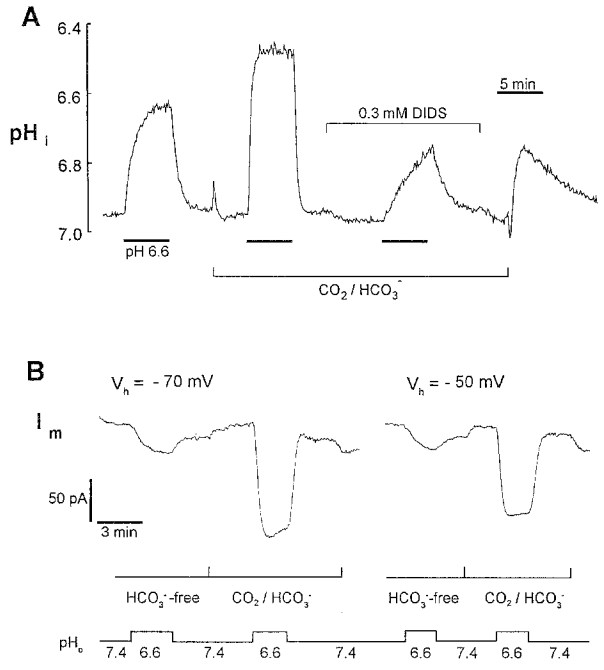


Fig. 7 The reduction of external pH from 7.4 to 6.6 in the absence and presence of CO_2/HCO_3^- on pH_i (**A**) and on the membrane current (**B**). The large and rapid pH_i changes in CO_2/HCO_3^--buffered saline could be greatly reduced by the stilbene DIDS (**A**). Changes in extracellular pH (pH_o) are shown at the *bottom* of the figures

(Fig. 7A). The amplitude and rate of these pH_i shifts were considerably reduced by DIDS. At the end of a 4-min exposure to pH 6.6, the pH_i was 6.75±0.09 (n = 5) in nominally CO_2/HCO_3^--free, PIPES-buffered saline, 6.54±0.08 (n = 5) in CO_2/HCO_3^--buffered saline, and 6.87±0.11 (n = 4) in the presence of CO_2/HCO_3^- and DIDS (0.3 mM). This suggests that the rapid intracellular acidification upon reduction of external pH to 6.6 was due to HCO_3^- efflux, and the rapid intracellular alkalinization upon restoring the external pH to 7.4 was due to HCO_3^- influx. Both changes in pH_i were independent of external Cl^- (not shown).

In voltage-clamp mode, reduction of the external pH to 6.6 evoked a much larger inward current in the presence of CO_2/HCO_3^- (–183±37 pA; n = 5) than in its nominal absence (–23±5 pA; n = 5; Fig. 7B) at a holding potential of –70 mV. When the membrane holding potential was shifted to more positive potentials, e.g. from –70 mV to –50 mV, the inward current induced upon the reduction of the external pH to 6.6 was reduced in the presence of CO_2/HCO_3^-, on average to –112±44 pA (n = 3; Fig. 7B). This indicates the dependence of this HCO_3^--dependent inward current on the membrane potential.

The results are consistent with a reversal of electrogenic HCO_3^- transport in the astrocyte membrane; reducing external pH and the HCO_3^- concentration would drive HCO_3^- out of the cells; this would generate an inward current, which would decrease at more positive potentials.

Discussion

The present results, using the fluorescent dye BCECF to measure pH_i and the whole-cell patch-clamp configuration to measure the membrane current, show that external Na^+ is essential for pH_i regulation in cerebellar astrocytes in culture. Our experiments could not reveal any Na^+-independent mechanism of pH_i regulation in these cells, as has been suggested by other studies on various types of mammalian astrocytes [5, 25, 31]. From our study we conclude that there is one HCO_3^--independent process, and at least one HCO_3^--dependent process that both contribute to the pH_i regulation in these astrocytes. These are an amiloride-sensitive Na^+/H^+ exchanger and an electrogenic Na^+-HCO_3^- cotransporter.

The main evidence for a Na^+/H^+ exchanger was the intracellular acidification after the addition of amiloride and after the removal of external Na^+ in the absence of CO_2/HCO_3^-. The results confirm the existence of a Na^+/H^+ exchanger in astrocytes, as has been shown by other studies in various mammalian astrocytes [5, 10, 21, 22], and other types of glial cells [2, 13, 19, 20].

The electrogenic Na^+-HCO_3^- cotransporter

Our experiments give evidence for the presence of an electrogenic Na^+-HCO_3^- cotransporter in rat cerebellar astrocytes by the following criteria: (1) the recovery from an intracellular acidification in CO_2/HCO_3^- was dependent upon external Na^+, and partly inhibited by DIDS; (2) the intracellular acidification evoked by amiloride could be partly reversed by CO_2/HCO_3^-; (3) the removal of CO_2/HCO_3^- was followed by a prominent, transient pH_i decrease, which was suppressed by the stilbene DIDS, but not in Cl^--free saline; (4) both HCO_3^--dependent pH_i changes were accompanied by outward and inward currents, which were greatly reduced or even reversed by the removal of external Na^+; (5) reduction of the external pH, and hence the HCO_3^- concentration, resulted in a rapid intracellular acidification and membrane potential-dependent inward currents, consistent with the reversal of an electrogenic Na^+-HCO_3^- cotransporter. The association of these membrane currents with the Na^+- and HCO_3^--dependent pH_i changes suggests that the stoichiometry of the Na^+-HCO_3^- cotransporter is either 2 or 3 HCO_3^-: 1 Na^+, and that therefore the pH_i regulation in astrocytes is partly voltage dependent.

After its first description in epithelial kidney cells [3], this carrier has been reported for many epithelial cells (for review see [4]). In most epithelial cells this cotransporter operates with a stoichiometry of 3 HCO_3^-: 1 Na^+, and hence extrudes Na^+ and HCO_3^-. Recent evidence suggests, however, that the stoichiometry may depend upon the direction of net transport [26].

In a variety of glial cells, a Na^+-HCO_3^- cotransporter has been described, in most of them with evidence for an electrogenic nature of this cotransport [1, 11, 13, 24]. In some of these studies, the stoichiometry and the revers-

ibility of the cotransporter has been worked out. In leech giant glial cells, the stoichiometry of the Na^+-HCO_3^- cotransporter is 2 HCO_3^-: 1 Na^+ [14, 23], whereas in salamander Müller glial cells, the stoichiometry was found to be 3 HCO_3^-: 1 Na^+ [24], as in most epithelial cells. From our present experiments, a definite stoichiometry of the electrogenic Na^+-HCO_3^- cotransporter in rat cerebellar astrocytes cannot be given. This would need a more rigorous analysis of the voltage dependence of HCO_3^--induced currents and pH_i changes, which is presently under investigation.

The main functional significance of an electrogenic Na^+-HCO_3^- cotransporter in glial cells is that pH_i regulation and possibly also regulation of extracellular pH becomes dependent upon the glial membrane potential. Changes in the extracellular K^+ concentration, which would de- or hyperpolarize the glial cell membrane, would lead to pH_i changes due to stimulation of inward or outward going Na^+-HCO_3^- cotransport across the glial membrane. The transport of base equivalents could also change the pH in the extracellular spaces; extracellular acidification or alkalinization may be due to uptake or secretion of HCO_3^- into or out of glial cells, respectively [11]. Similar effects have been proposed and discussed in other studies on glial control of pH in nervous systems [11, 12, 24, 27].

CO_2/HCO_3^--dependent pH_i changes

The recovery from the intracellular acidification upon addition of CO_2/HCO_3^- appeared to be due to at least two, if not three mechanisms, the Na^+/H^+ exchanger, the Na^+-HCO_3^- cotransporter and, possibly, a Na^+-dependent Cl^-/HCO_3^- exchanger. There was no pH_i regulation in the absence of external Na^+. pH_i recovery from acidification was much faster in the presence than in the absence of CO_2/HCO_3^-. A large increase of pH_i was observed upon addition of CO_2/HCO_3^- in the presence of amiloride, which inhibits Na^+/H^+ exchange, but not HCO_3^--dependent pH_i regulation, and which brought the pH_i close to the initial alkaline value in the absence of amiloride and CO_2/HCO_3^-. This may indicate that Na^+/H^+ exchange and HCO_3^--dependent acid extrusion play a similarly important role in determining the resting pH_i.

There was a conspicuous intracellular acidification following the removal of CO_2/HCO_3^- in these cultured astrocytes. This acidification was blocked by DIDS, but was unaffected by amiloride and by the removal of Cl^-, suggesting that it was due to HCO_3^- outward transport. The concomitant transient inward current was consistent with the net extrusion of negative charges.

The recovery from this acidification following the removal of CO_2/HCO_3^- was suppressed by amiloride, and hence presumably due to Na^+/H^+ exchange. Indeed, the rates of pH_i recovery from the acidification after CO_2/HCO_3^- removal and after CO_2/HCO_3^- addition in the presence of DIDS were similar, but significantly slower than in normal, CO_2/HCO_3^--buffered saline (see above). The entry and exit of CO_2 produced a very fast intracellular acidification and alkalinization, respectively, presumably due to the activity of carbonic anhydrase in these cells. These pH_i changes were apparently counteracted by a Cl^--independent HCO_3^- influx and efflux, e.g. via Na^+-HCO_3^- cotransport and, possibly to some extent, via a HCO_3^--conductive pathway. In the absence of external Na^+, however, addition of CO_2/HCO_3^- never produced even a transient intracellular alkalinization, as would be expected if the cells had a significant HCO_3^- conductance.

The CO_2/HCO_3^--induced membrane currents

The outward current upon addition, and the inward current upon removal, of CO_2/HCO_3^- were partly transient, as would be expected since changes in pH_i and the intracellular HCO_3^- concentration would alter the ionic gradients. The CO_2/HCO_3^--dependent membrane currents might also be due to a HCO_3^- conductance of the cell membrane. At steady-state, where the electrochemical gradient of HCO_3^- is outwardly directed (E_{HCO3^-} positive to E_m), a net inward current would be expected in CO_2/HCO_3^--buffered saline. In our experiments, however, there was always a maintained net outward current even during prolonged exposure to CO_2/HCO_3^-.

The transient inward current upon removal of CO_2/HCO_3^- was consistent with the simultaneous, prominent pH_i fall, supporting the exit of base equivalents via a HCO_3^- conductance pathway and/or the electrogenic Na^+-HCO_3^- cotransporter.

The reversal of the current in the absence of external Na^+, as observed in some experiments, is consistent with the electrogenic outward transport of Na^+ and HCO_3^- under the experimental conditions, when intracellular Na^+ should never decline to much less than 5 mM, which was the Na^+ concentration in the patch electrode. The Na^+ dependence of the CO_2/HCO_3^--induced current cannot be reconciled with a HCO_3^- conductance. However, in most experiments there was only a *reduction* of the outward current upon addition of CO_2/HCO_3^- in Na^+-free saline. This suggests that there may also be a Na^+-independent component of the outward current in some cells, which could be due to some HCO_3^- conductance.

The correlation of the HCO_3^-- and Na^+-dependent membrane current with the pH_i changes measured with BCECF can only be qualitative for mainly two reasons. First, the currents were measured in a single whole-cell clamp, while pH_i was recorded from a large group of intact cells. Second, the patch pipette solution was buffered, and dialysis of the cell from which recordings were made would help to buffer the cytoplasm, although the buffer capacity of 10 mM HEPES added would presumably increase the total cytoplasmic buffering power in the presence of CO_2/HCO_3^- by less than 30%. Nevertheless, the membrane current data provide essential evidence for the electrogenicity of the pH_i-regulating process, and hence electrogenic Na^+-HCO_3^- cotransport may have remained undetected in studies recording pH_i only [5, 10, 22].

Acknowledgements This study was supported by the Deutsche Forschungsgemeinschaft, SFB 246, TP C7.

References

1. Astion ML, Orkand RK (1988) Electrogenic Na^+/HCO_3^- co-transport in neuroglia. Glia 1:355–357
2. Astion ML, Chvatal A, Orkand RK (1991) Further studies of electrogenic Na^+/HCO_3-cotransport in glial cells of Necturus optic nerve: regulation of pH_i. Glia 4:461–468
3. Boron WF, Boulpaep EL (1983) Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO_3^- transport. J Gen Physiol 81:53–94
4. Boron WF, Boulpaep EL (1989) The electrogenic Na/HCO_3 cotransporter. Kidney Int 36:392–402
5. Boyarski G, Ransom B, Schlue WR, Davis MBE, Boron WF (1993) Intracellular pH regulation in single cultured astrocytes from rat forebrain. Glia 8:241–248
6. Cabantchik ZI, Rothstein A (1972) The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulphonic stilbene derivatives. J Membr Biol 10:311–330
7. Chesler M, Kaila K (1992) Modulation of pH by neuronal activity. Trends Neurosci 15:396–402
8. Chesler M, Kraig RP (1987) Intracellular pH of astrocytes increase rapidly with cortical stimulation. Am J Physiol 253:R666–R670
9. Chesler M, Kraig RP (1989) Intracellular pH transients of mammalian astrocytes. J Neurosci 9:2011–2019
10. Chow SY, Yen-Chow YC, White HS, Woodbury DM (1991) pH regulation after acid load in primary cultures of mouse astrocytes. Dev Brain Res 60:69–78
11. Deitmer JW (1991) Electrogenic sodium-dependent bicarbonate secretion by glial cells of the leech central nervous system. J Gen Physiol 98:637–655
12. Deitmer JW (1992) Evidence for glial control of extracellular pH in the leech central nervous system. Glia 5:43–47
13. Deitmer JW, Schlue WR (1987) The regulation of intracellular pH by identified glial cells and neurones in the central nervous system of the leech. J Physiol (Lond) 388:261–283
14. Deitmer JW, Schlue WR (1989) An inwardly directed electrogenic sodium-bicarbonate co-transport in leech glial cells. J Physiol (Lond) 411:179–194
15. Deitmer JW, Szatkowski M (1990) Membrane potential dependence of intracellular pH regulation by identified glial cells in the leech central nervous system. J Physiol (Lond) 421:617–631
16. Fenwick EM, Marty A, Neher E (1982) A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. J Physiol (Lond) 331:577–597
17. Fischer G (1984) Growth requirements of immature astrocytes in serum-free hormonally defined media. J Neurosci Res 12:543–552
18. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100
19. Jean T, Frelin C, Vigne P, Lazdunski M (1986) The Na^+/H^+ exchange system in glial cell lines. Properties and activation by an hyperosmotic shock. Eur J Biochem 160:211–219
20. Kettenmann H, Schlue WR (1988) Intracellular pH regulation in cultured mouse oligodendrocytes. J Physiol (Lond) 406:147–162
21. Mellergard PE, Ouyang YB, Siesjö BK (1992) Regulation of intracellular pH in cultured astrocytes and neuroblastoma cells: dependence on extracellular pH. Can J Physiol Pharmacol 70:S293–S300
22. Mellergard PE, Ouyang YB, Siesjö BK (1993) Intracellular pH regulation in cultured rat astrocytes maintained in CO_2/HCO_3^- containing media. Exp Brain Res 95:371–380
23. Munsch T, Deitmer JW (1994) Sodium-bicarbonate cotransport current in identified leech glial cells. J Physiol (Lond) 474:43–53
24. Newman EA (1991) Sodium-bicarbonate cotransport in retinal Müller (glial) cells of the salamander. J Neurosci 11:3972–3983
25. Pappas CA, Ransom BR (1993) A depolarization-stimulated, bafilomycin-inhibitable H^+ pump in hippocampal astrocytes. Glia 9:280–291
26. Planelles G, Thomas SR, Anagnostopoulos T (1993) Change of apparent stoichiometry of proximal tubule Na^+-HCO_3-cotransport upon experimental reversal of its orientation. Proc Natl Acad Sci USA 90:7406–7410
27. Ransom B (1992) Glial modulation of neural excitability mediated by extracellular pH: a hypothesis. In: Yu ACH, Hertz L, Norenberg MD, Sykova E, Waxman SG (eds) Progress in brain research, vol 94. Elsevier pp 37–46
28. Rose CR, Deitmer JW (1994) Stimulus-evoked changes of extra- and intracellular pH in the leech central nervous system. I. Bicarbonate dependence (submitted)
29. Sykova E (1983) Extracellular K^+ accumulation in the central nervous system. Prog Biophys Mol Biol 42: 135–189
30. Thomas JA, Buchsbaum RN, Zimniak A, Racker E (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry 18:2210–2218
31. Wuttke W, Walz W (1990) Sodium- and bicarbonate-independent regulation of intracellular pH in cultured mouse astrocytes. Neurosci Lett 117:105–110