Electrogenic Sodium-dependent Bicarbonate Secretion by Glial Cells of the Leech Central Nervous System

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ABSTRACT The ability to move acid/base equivalents across the membrane of identified glial cells was investigated in isolated segmental ganglia of the leech *Hirudo medicinalis*. The intracellular pH (pH_i) of the glial cells was measured with double-barreled, neutral-ligand, ion-sensitive microelectrodes during step changes of the external pH (pH_o 7.4–7.0). The rate of intracellular acidification after the decrease in extracellular pH (pH_o) was taken as a measure of the rate of acid/base transport across the glial membrane. Taking into account the total intracellular buffering power, the maximum rate of acid/base flux was 0.4 mM/min in CO_2/HCO_3^--free saline, and 3.92 mM/min in the presence of 5% CO_2/10 mM HCO_3^-, suggesting that the acid/base flux was dependent upon HCO_3^-. The rate of acid influx/base efflux increased both with the external HCO_3^- concentration and with increasing pH_i (and hence HCO_{3i}^-). This suggested that the decrease in pH_i was due to HCO_3^- efflux. The rapid decrease of pH_i was accompanied by a HCO_3^--dependent depolarization of the glial membrane from -74 ± 5 mV ($n = 20$) to -54 ± 7 mV ($n = 13$). Both this depolarization and the rate of intracellular acidification were greatly reduced by the anion exchange inhibitor 4,4-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS; 0.3–0.5 mM), but were not affected by the removal of external Cl^-. Reduction of the external Na^+ concentration to one-tenth normal affected the rate of intracellular acidification only in the presence of CO_2/HCO_3^-: the rate increased within the first 3–5 min after lowering external Na^+; after longer exposures in low external Na^+ the rate decreased, presumably due to depletion of intracellular Na^+. Amiloride (1 mM), which inhibits the Na^+-H^+ exchange in these cells, had no effect on the rate of intracellular acidification. The intracellular Na activity (aNa_i) of the glial cells was measured to be 5.2 ± 1.0 mM ($n = 8$) in CO_2/HCO_3^--free saline; aNa_i increased to 7.3 ± 2.2 mM ($n = 8$) after the addition of 5% CO_2/24 mM HCO_3^-. Upon a change in pH_o to 7.0 in the presence of CO_2/HCO_3^-, aNa_i decreased by an average of 2 ± 1.1 mM ($n = 5$); in CO_2/HCO_3^--free saline external acidification produced a transient increase in aNa_i. It is concluded that, in the presence of CO_2/HCO_3^-, the rate of intracellular acidification in glial cells is dominated by an outwardly directed, electrogenic

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Na^+-HCO_3^- cotransport. Neurons, which do not possess this cotransporter, acidify at much lower rates under similar conditions. Since the Na^+-HCO_3^- cotransporter is reversible, glial cells might play a crucial role in dampening ("muffling") acid and base transients in the extracellular spaces of the nervous system by active, Na^+-dependent secretion and uptake of HCO_3^-.

INTRODUCTION

Living cells usually maintain an alkaline intracellular pH (pH_i) with respect to the H^+ electrochemical equilibrium by actively removing acid equivalents across the cell membrane. This has been extensively studied in neurons (for references see Roos and Boron, 1981; Thomas, 1984; Chesler, 1990). The main mechanisms involved are coupled ion transport processes across the membrane, such as Na^+-H^+ and Na^+-dependent Cl^--HCO_3^- exchange.

In glial cells, similar mechanisms of pH regulation have recently been reported, as well as a DIDS-sensitive electrogenic Na^+-HCO_3^- cotransporter (Deitmer and Schlue, 1987, 1989; Astion and Orkand, 1988; Kettenmann and Schlue, 1988). Upon introduction of CO_2/HCO_3^-, this Na^+-HCO_3^- cotransport is inwardly directed and hence alkalinizes the glial cytoplasm. Since more than one HCO_3^- ion is transported per Na^+ ion, the intracellular alkalinization is accompanied by a DIDS-sensitive membrane hyperpolarization. In leech neuropile glial cells, the stoichiometry of this cotransporter was determined to be 1 Na^+:2 HCO_3^- (Deitmer and Schlue, 1989), and it was shown to be sensitive to membrane potential changes (Deitmer and Szatkowski, 1990).

Much less is known, however, about the transport of acid equivalents *down* the H^+ electrochemical gradient. With respect to the Na^+-HCO_3^- cotransporter in glial cells, this might be achieved by the reversal of the cotransporter. Indeed, in many epithelial cells electrogenic Na^+-HCO_3^- cotransport is outwardly directed in its normal mode (cf. Boron and Boulpaep, 1989). In the proximal tubule of the salamander kidney, where Na^+-HCO_3^- cotransport was first described at the basolateral membrane (Boron and Boulpaep, 1983), this produces an intracellular acidification of the epithelial cells and serves to reabsorb HCO_3^- into the blood.

Acid uptake into, or base secretion from cells might be of importance in the nervous system, where considerable extracellular acid transients may occur during neuronal activity (Kraig, Ferreira-Filho, and Nicholson, 1983; Mutch and Hansen, 1984; Somjen, 1984; Krishtal, Osipchuk, Shelest, and Smirnoff, 1987; Chesler and Chan, 1988; Chvátal, Jendelová, Kriz, and Syková, 1988; Rice and Nicholson, 1988; Walz, 1989). This, in turn, may affect the activity of nerve cells (Fukuda and Loeschcke, 1977; Jarolimek, Misgeld, and Lux, 1990) and glial cells (Walz and Hinks, 1987) by influencing channels, carriers, and synaptic receptors of these cells.

Glial cells, which play an important role in the regulation of the K^+ microenvironment in the nervous system (Hertz, 1965, 1990; Nicholson, 1983; Walz, 1989), may also be good candidates to help control the pH of the extracellular (interstitial) spaces. This would require a mechanism of rapid acid/base influx or efflux across the glial membrane. In previous studies, effective HCO_3^- uptake by glial cells was shown to be due to inwardly directed Na^+-HCO_3^- cotransport; this may produce acid transients or counteract alkaline transients in the extracellular spaces. On the other

hand, a reversed mode of the cotransporter might be activated by an extracellular acidification, and hence be able to remove acid from the interstitial spaces. In this study I have tried to test this possibility by measuring the pH_i and the intracellular Na activity (aNa_i) of identified glial cells in the neuropile of the leech central nervous system. The results suggest that under appropriate thermodynamic conditions, the electrogenic Na^+-HCO_3^- cotransport in the glial membrane can be directed outwardly. This effective secretion of base equivalents into the extracellular spaces might be significant for acid/base homeostasis in the brain.

METHODS

The experiments were performed on the anterior or posterior glial cell in the neuropile of segmental ganglia of the leech *Hirudo medicinalis*. The preparations and dissection procedures used to isolate single ganglia have been described before (Schlue and Deitmer, 1980; Deitmer and Schlue, 1981). Isolated ganglia were pinned by their connectives, ventral side upward, to the silicone rubber base of a small experimental chamber (volume, 0.1 ml). The ganglia were superfused with saline at a rate of ~2 ml/min. The selection and identification of the glial cells and neurons followed criteria described previously (Deitmer and Schlue, 1981, 1987). All experiments were performed 3–12 times, each type with essentially similar protocols, at room temperature (22–25°C).

Physiological Solutions

The CO_2/HCO_3^--free saline had the following composition (in mM): 85 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), adjusted to pH 7.4 with 4.5–5 mM NaOH. When the pH was changed to 7.0, 10 mM MOPS (3-(*N*-morpholino)propanesulfonic acid) instead of HEPES was used as a buffer. Solutions containing CO_2/HCO_3^- had the same amount of HEPES or MOPS, and were bubbled with either 1% or 5% CO_2; 2 and 5 mM HCO_3^- or 10 and 24 mM HCO_3^- were added to make a pH of 7.0 and 7.4, respectively. Equivalent amounts of NaCl were replaced by $NaHCO_3$ to maintain isotonicity. Reduction of external Na^+ was achieved by replacing NaCl by *N*-methyl-D-glucamine (NMDG) neutralized with HCl and/or CO_2 as appropriate. In some experiments the stilbene DIDS (4,4-diisothiocyanatostilbene-2,2'-disulfonic acid, 0.3–0.5 mM) or the diuretic amiloride (1 mM) was applied directly to the saline shortly before use.

Microelectrodes

"Side-by-side" double-barreled borosilicate microelectrodes were used for recording pH or Na^+ and the reference (membrane) potential. The electrodes were pulled in two stages and were silanized essentially as described by Borelli, Carlini, Dewey, and Ransom (1985): a drop of a 5% tri-*N*-butylchlorosilane in 99.9% pure carbon tetrachloride solution was filled into the prospective ion-selective barrel. The pipette was then baked on a hot plate at 460–475°C for 4.5–5 min.

For the pH-sensitive electrodes, H^+ cocktail (Fluka 95291) was backfilled into the tip of the silanized barrel, and then filled up with 0.1 M Na-citrate, pH 6.0. The reference barrel was filled with 3 M KCl. The electrodes were calibrated in leech saline buffered to 7.4 with HEPES, 7.0 with MOPS, and 6.4 with PIPES (piperazine-*N*,*N'*bis(2-ethanesulfonic acid) (Fig. 1 *A*). They responded on average with a 52-mV unit change in pH. The sensitivity to CO_2/HCO_3^- was tested for each electrode, and a response of up to 5 mV (i.e., <0.1 pH unit) was accepted. Most electrodes used responded with <3 mV to 5% or 1% CO_2 (Fig. 1 *A*).

For Na^+-sensitive electrodes, Na^+ cocktail was produced by mixing the Na^+ ionophore VI (Fluka 71739), 2-*N*-phenyl-octyl-ether (o-NPOE; Fluka 73732) and Na-tetraphenylborate

(Fluka 72018) in wt % at a ratio 10:89.5:0.5. This mixture was backfilled into the silanized barrel, and then filled up with 0.1 M NaCl + 10 mM MOPS, pH 7.0. The reference barrel was filled with 3 M KCl. The electrode was calibrated in salines with different Na^+ concentrations at constant ionic background with NMDG or K^+ as the Na^+ substitute (Fig. 1 *B*). The electrodes responded on average with 53 mV between 90 and 9 mM Na^+ concentration, corresponding to 67.5 and 6.75 mM Na activity, using an activity coefficient of 0.75 for Na^+. At lower Na^+ concentrations the electrode response was nearly linear down to 4.5 mM (3.4 mM activity), but 3–5 mV less when Na^+ was replaced by K^+ instead of NMDG (Fig. 1 *B*). All measurements of Na^+ are presented as Na activity.

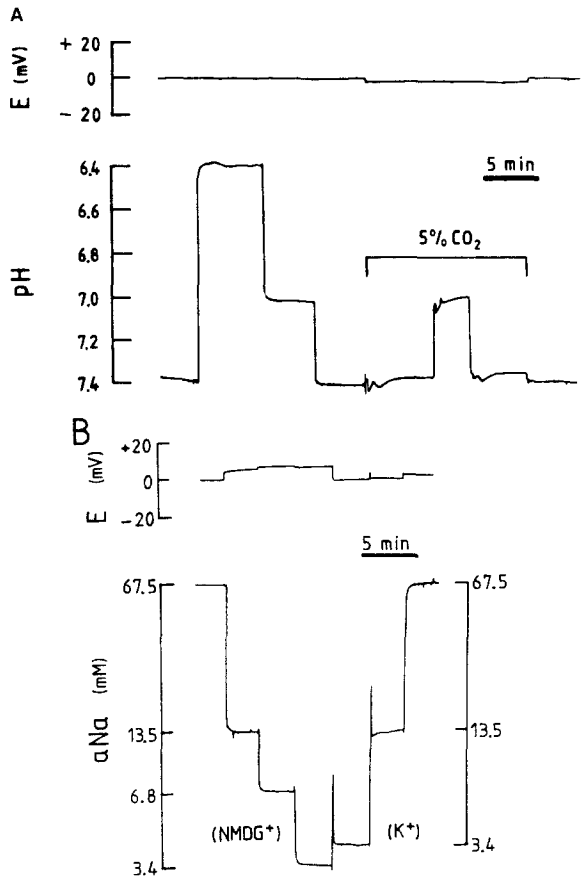

FIGURE 1. Calibration of a pH-sensitive (*A*) and a Na^+-sensitive (*B*) microelectrode. The pH-sensitive microelectrode was tested in leech salines at pH 7.4, 6.4, and 7.0, and back to 7.4 in CO_2/HCO_3^--free saline and in saline containing 5% CO_2 at pH 7.4 (24 mM HCO_3) and pH 7.0 (10 mM HCO_3^-). The Na^+-sensitive microelectrode was tested in leech saline containing different Na^+ concentrations (here converted to Na activities), when Na^+ was replaced by *N*-methyl-D-glucamine (NMDG; left part, when the Na^+ concentration was lowered) or by K^+ (right part, when the Na^+ concentration was restored).

Recording

The electrical arrangements were the same as described previously (Deitmer and Schlue, 1981, 1987). Each channel of the double-barreled microelectrode was connected to one input of a differential electrometer (FD-223; World Precision Instruments, Inc., New Haven, CT). The electrometer outputs were displayed on an oscilloscope and recorded on tape and/or on chart paper for direct display.

RESULTS

The standard protocol used in the present study was to change the pH of the saline from 7.4 to 7.0 for 5–15 min, either in nominal CO_2/HCO_3^--free saline or in a CO_2-gassed saline containing different HCO_3^- concentrations. An experiment of this type with a pH-sensitive microelectrode in a neuropile glial cell is shown in Fig. 2. The external HCO_3^- concentration was first reduced from 24 to 10 mM at constant pressure of 5% CO_2 with a concomitant decrease of the pH to 7.0, and then from 24 mM HCO_3^- (5% CO_2) to nominal CO_2/HCO_3^--free saline with a pH of 7.0. The maximum rates of intracellular acidification were determined as the initial ΔpH_i/min; they were 0.052 and 0.065 pH units/min, respectively. A similar rate of intracellular acidification occurred after the removal of CO_2/HCO_3^- at constant pH (7.4). This suggests that it was the decrease of the external HCO_3^- concentration which induced the rapid intracellular acidification in these glial cells.

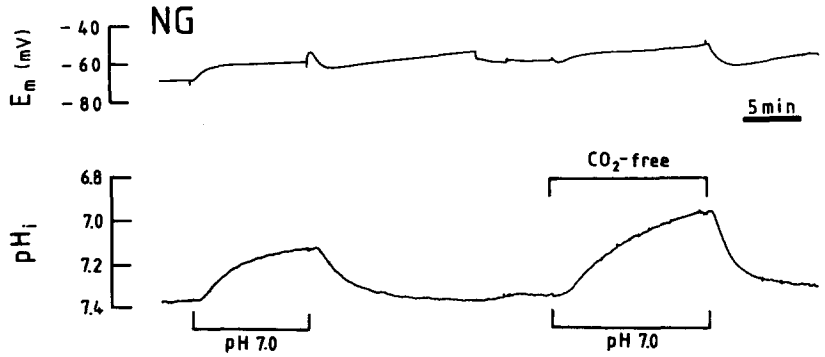

FIGURE 2. Changes of pH_i (*lower trace*) and membrane potential (E_m; *upper trace*) of a neuropile glial cell after the reduction of external pH from 7.4 to 7.0 in a saline bubbled with 5% CO_2 and with concomitant removal of CO_2/HCO_3^-.

The steady-state values of pH_i were 7.25 ± 0.12 (n = 12) in 5% CO_2/24 mM HCO_3^- (pH_o 7.4), and 7.05 ± 0.1 (n = 9) in 5% CO_2/10 mM HCO_3^- (pH_o 7.0). The glial membrane reversibly depolarized after the reduction of the external HCO_3^- concentration, on average by 20 mV; i.e., from −74 ± 5 mV (n = 20) to −54 ± 7 mV (n = 13; Table I).

After restoring the external pH and/or the HCO_3^- concentration, the pH_i increased rapidly to its initial value. The maximum rates of this pH_i recovery were 0.08 and 0.17 mM/min, and were accompanied by a membrane hyperpolarization. It was shown previously that addition of HCO_3^- to the external solution stimulates an inwardly directed, electrogenic Na^+-HCO_3^- cotransport (Deitmer and Schlue, 1989).

The equilibrium potential of H^+ and HCO_3^- in CO_2/HCO_3^--containing salines changed from −9 mV at pH_o 7.4 to +2 mV at pH_o 7.0 (Table I). With the different membrane potentials in the high and low HCO_3^- salines, the electrochemical gradients for H^+ and HCO_3^- were 56 and 65 mV, respectively. In CO_2/HCO_3^--free

saline the H^+/HCO_3^- equilibrium potential was −25 mV, resulting in an electrochemical gradient for H^+/HCO_3^- of 40 mV.

Acid/Base Fluxes across the Membrane

To convert the rate of pH_i change into fluxes of acid or base equivalents across the membrane, the intracellular buffer capacity has to be taken into account. The mean intrinsic buffer capacity, β_i, of the glial cells had been determined previously by the ammonium pulse technique to be 25 mM in CO_2/HCO_3^--free, HEPES-buffered saline (Deitmer and Schlue, 1987). A similar value was found using the weak acid (propionic acid) method (after Szatkowski and Thomas, 1989). In the presence of CO_2/HCO_3^-, a CO_2-induced buffer capacity, β_{CO_2}, is added, which is given in first approximation by

$$\beta_{CO_2} = 2.3[HCO_3^-]_i \quad (1)$$

The intracellular HCO_3^- concentration was calculated with the Henderson-Hasselbalch equation, using the value of pH_i measured with the ion-sensitive electrode. The

TABLE I

pH_i, Na^+, and HCO_3^- in Salines Containing Different HCO_3^- Concentrations, and the Corresponding Equilibrium Potentials and Membrane Potentials of the Neuropile Glial Cells

Saline	pH_i	$HCO_{3\,i}^-$	aNa_i	E_m	E_H	E_{Na}
		mM	*mM*	*mV*	*mV*	*mV*
CO_2-free	6.97 ± 0.1 (n = 13)	<0.1	5.2 ± 1.0 (n = 8)	−65 ± 4 (n = 21)	−25	+65
5% CO_2, pH 7.4	7.25 ± 0.12 (n = 12)	17	7.35 ± 2.2 (n = 8)	−74 ± 5 (n = 20)	−9	+56
5% CO_2, pH 7.0	7.05 ± 0.1 (n = 9)	11	5.3 ± 2.0 (n = 5)	−54 ± 7 (n = 13)	+2	+65

total buffer capacity, β_t, is the sum of β_i and β_{CO_2}. At a mean pH_i of 7.25 in the presence of 5% CO_2/24 mM HCO_3^-, the intracellular HCO_3^- concentration was 17 mM (Table I). This resulted in a β_{CO_2} of 39 mM and a β_t of 64 mM. With the rate of pH_i change and β_t known, the acid flux, J_H, or the base flux in opposite direction, J_{HCO_3}, are given by

$$J_H = -J_{HCO_3} = \Delta pH_i/\text{min} \times \beta_t \quad (2)$$

The acid/base flux ($J_{ac/b}$; Table II) was determined using the initial maximum rate of pH_i change at the first 1–2 min of the change to a saline with different pH. The maximum rates of pH_i change upon reduction of the external HCO_3^- concentration, as shown in Fig. 2, would indicate an acid influx of 4.0 mM/min (10 mM HCO_3^-, pH 7.0) and 5.0 mM/min (HCO_3^--free, pH 7.0), or a base efflux of the same values. In comparison, the change from a saline containing 5% CO_2/24 mM HCO_3^- (pH 7.4) to CO_2/HCO_3^--free saline (pH 7.4) induced a maximum acid influx/base efflux of 3.98 mM/min in the same experiment (not shown).

TABLE II

Rate of Intracellular Acidification (ΔpH_i/min) and Acid/Base Flux ($J_{ac/b}$) in Neuropile Glial Cells and Retzius Neurones after a Change of External pH from 7.4 to 7.0 in the Presence of Different HCO_3^- Concentrations, and in the Presence of the Stilbene DIDS (0.3–0.5 mM)

Saline	Glia		Neurone	
	ΔpH_i/min	$J_{ac/b}$	ΔpH_i/min	$J_{ac/b}$
CO_2-free	0.015 ± 0.004 (n = 8)	0.38 ± 0.1 (n = 8)	0.01 ± 0.004 (n = 12)	0.2 ± 0.08 (n = 12)
5% CO_2	0.07 ± 0.02 (n = 20)	3.92 ± 1.1 (n = 20)	0.022 ± 0.009 (n = 14)	1.3 ± 0.5 (n = 14)
5% CO_2 + DIDS	0.022 ± 0.008 (n = 8)	0.55 ± 0.2 (n = 8)	—	—

HCO_3^- Dependence of Acid/Base Fluxes

Fig. 3 shows an experiment where the external pH was repeatedly reduced to 7.0 at different HCO_3^- concentrations. The rate of intracellular acidification decreased with lower HCO_3^- concentrations, and it was greatly reduced in nominally CO_2/HCO_3^--free saline. In the experiments shown, the maximum (initial) rate of pH_i change was 0.055 pH units/min in the presence of 5% CO_2/10 mM HCO_3^-, which corresponds to an acid influx of 3.8 mM/min (or a base efflux of −3.8 mM/min). In the presence of 1% CO_2/2 mM HCO_3^- the maximum rate of pH_i change was 0.047 pH units/min, which was calculated to be equivalent to an acid/base flux of (−) 2.3 mM/min. In the nominal absence of CO_2/HCO_3^- (where the HCO_3^- concentration of the saline amounts to ~60 μM at pH_o 7.0 due to the equilibration of air-CO_2 in the solutions), the rate of maximum pH_i change was 0.018 pH units/min, corresponding to an acid/base flux of (−) 0.45 mM/min across the glial membrane. This is a flux rate about five times lower than in the presence of 1% CO_2/2 mM HCO_3^-, and more than eight times lower than in the presence of 5% CO_2/10 mM HCO_3^-.

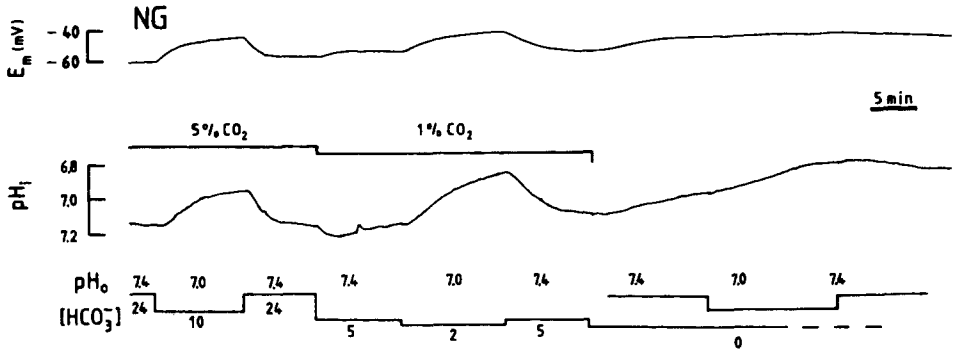

FIGURE 3. Changes in pH_i and membrane potential after the reduction of external pH at different external concentrations of CO_2/HCO_3^- (5, 1, and 0% CO_2). Note the different initial rates of the intracellular acidifications.

This suggests that the rates of intracellular acidification were HCO_3^- dependent, and reflect HCO_3^- efflux rather than H^+ influx. Accordingly, they were expected to vary with pH_i, and hence with the intracellular HCO_3^- concentration, too. This is evident when the acid/base fluxes as calculated from the experiment shown in Fig. 3 were plotted against pH_i (Fig. 4). There was a strong dependence of the transmembrane transport of acid/base equivalents on both the external HCO_3^- concentration and pH_i. The plot also shows that the different rates of acid/base flux were not simply due to the different values of the initial pH_i in the various salines before changing to pH_o 7.0, when the rates of intracellular acidification were measured.

The mean maximum acid/base flux calculated from the initial pH_i changes was 3.92 ± 1.1 mM/min (n = 20) in the presence of 5% CO_2/10 mM HCO_3^-, as compared with 0.38 ± 0.1 mM/min (n = 8) in the nominal absence of CO_2/HCO_3^- (Table II). This indicated that in the presence of CO_2/HCO_3^- the rate of intracellular acidification was dominated by >90% by HCO_3^- efflux.

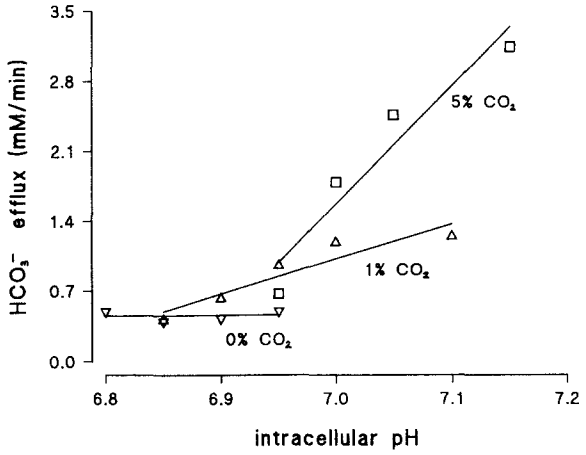

FIGURE 4. Dependence on pH_i of the rate of acid equivalent influx plotted as HCO_3^- efflux at different external CO_2/HCO_3^- concentrations as indicated.

Removal of external Cl^- (exchanged by glucuronate) did not affect the rate of intracellular acidification. Thus, the HCO_3^- efflux was not coupled to a Cl^- influx, as would be expected during stimulation of a Cl^--HCO_3^- exchanger.

Effect of Ethoxzolamide

An intracellular acidification produced by a HCO_3^- efflux might be dependent on the rate of HCO_3^- production in the cell due to carbonic anhydrase activity. This enzyme can be inhibited (irreversibly) by the membrane permeable ethoxzolamide (EZA) in leech glial cells and neurons (unpublished observations). Addition of 2 μM EZA tended to reduce the rate of intracellular acidification after lowering pH_o from 7.4 to 7.0 and the HCO_3^- concentration from 24 to 10 mM. The mean rate was decreased to 72 ± 20% (n = 7) in the presence of EZA. It should be emphasized, however, that while in some experiments the reduction of the rate of intracellular acidification, indicative of HCO_3^- efflux, was reduced by >50%, in three experiments the effect of EZA on this rate was <10%. In addition, the buffering power would be expected to change if the HCO_3^- production was slowed by EZA, which itself would enhance the

rate of intracellular acidification. Furthermore, the activity of carbonic anhydrase in other compartments (neurons and extracellular space) would also be inhibited by EZA, which makes it difficult to interpret the relatively small EZA effect. This is especially true since EZA also decreases the apparent buffer capacity of the extracellular spaces surrounding neurons and glial cells (Thomas, Coles, and Deitmer, 1991).

Effect of DIDS

In another type of experiment I tested whether the anion exchange inhibitor DIDS had an effect on the rate of intracellular acidification in the presence of CO_2/HCO_3^- (Fig. 5). After two exposures to pH_o 7.0 (Fig. 5 *A*), 0.5 mM DIDS was added to the

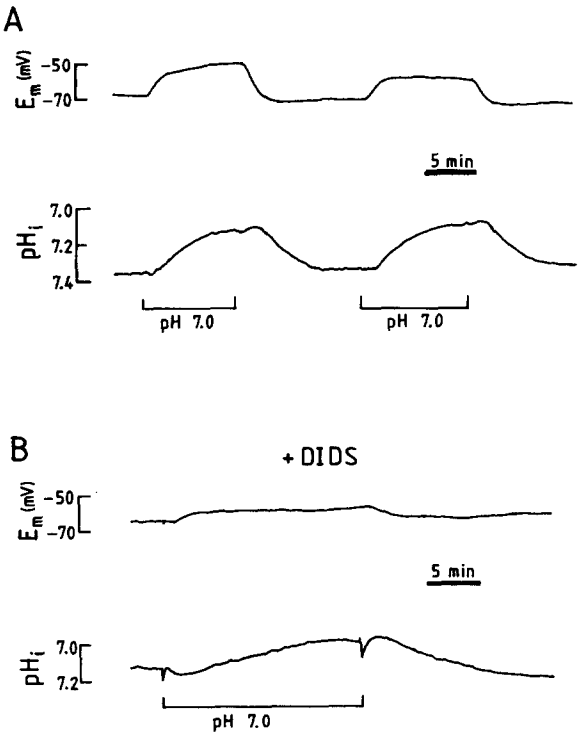

FIGURE 5. Effect of DIDS (0.5 mM) on the rate of intracellular acidification induced by lowering the external pH from 7.4 to 7.0 in a saline bubbled with 5% CO_2 containing 24 and 10 mM HCO_3^-, respectively. After two control measurements (*A*), DIDS was added 10 min before another exposure to pH 7.0 was performed (*B*).

saline for 10 min. This is sufficient to block the Na^+-HCO_3^+ cotransporter in these glial cells (Deitmer and Schlue, 1989; Deitmer and Szatkowski, 1990). The subsequent exposure to pH_o 7.0 induced a much smaller and slower intracellular acidification (Fig. 5 *B*).

The mean rate of intracellular acidification in the presence of DIDS was reduced to 0.022 ± 0.008 pH units/min (n = 8). Since the buffer capacity of the glial cytoplasm was also reduced by DIDS to values found in CO_2/HCO_3^--free salines (Deitmer, 1990, 1991), this rate of acidification indicated a maximum flux of acid/base across the glial membrane of 0.55 ± 0.2 mM/min. This value is close to the flux found in CO_2/HCO_3^--free saline. Thus, DIDS completely blocked the HCO_3^--dependent rate of

acidification; in other words, the HCO_3^- efflux from glial cells, induced by the reduction of external pH and HCO_3^- concentration, was largely DIDS sensitive. The membrane depolarization observed during the induced HCO_3^- efflux was also reduced by DIDS from 16 mV to 2–5 mV. This is in line with a DIDS-sensitive electrogenic efflux of HCO_3^-.

Since under all experimental conditions the gradient for HCO_3^- is directed outwardly, this HCO_3^- efflux might be due to a high HCO_3^- conductance of the glial membrane. The positive shift of the HCO_3^- electrochemical potential upon reducing the external HCO_3^- concentration from 24 to 10 mM briefly increases the HCO_3^- outward gradient further. The membrane depolarization observed during the intracellular acidification would be in line with a high HCO_3^- permeability.

Previous studies, however, did not indicate a high HCO_3^- permeability of the glial membrane (Deitmer and Schlue, 1989), although a high Cl^- conductance has been reported for leech glial cells (Ballanyi and Schlue, 1990; Munsch and Deitmer, 1990). Measurement of the input resistance indicated only a small change of membrane conductance (< 10%) when replacing a CO_2/HCO_3^--free saline with one containing 5% CO_2/24 mM HCO_3^- (Munsch, T., and J. W. Deitmer, unpublished observations). This suggests that the HCO_3^- conductance of the glial membrane would be rather low.

Alternatively, the high rate of HCO_3^- efflux might be due to coupled ion transport via a carrier such as the electrogenic Na^+-HCO_3^- cotransport in the glial membrane (Deitmer and Schlue, 1987, 1989), which was suggested to reverse from an inwardly directed mode to an outwardly directed mode under appropriate conditions (Deitmer and Szatkowski, 1990). If the outwardly directed cotransport was also electrogenic as was shown for the inwardly directed mode, its stimulation would cause a membrane depolarization, which indeed was observed in the present experiments. Therefore, the importance of the Na^+ gradient across the membrane for the rate of intracellular acidification was studied.

Dependence of the Acid/Base Flux on the Na^+ Gradient

If the efflux of HCO_3^- was coupled to Na^+, reduction of the external Na^+ concentration should alter the rate of intracellular acidification in the presence of CO_2/HCO_3^-. This was tested in two types of experiments.

First, the change of the external pH to 7.0 was briefly preceded by the reduction of the external Na^+ concentration. Since immediately after the reduction of the external Na^+ concentration Na^+ movement out of the cell is facilitated, any Na^+-coupled efflux should be accelerated. This was indeed the case, as shown in Fig. 6 *A*. The rate of intracellular acidification was 0.053 pH units/min in normal Na^+ (90 mM), indicating a HCO_3^- efflux rate of 2.5 mM/min, and 0.159 pH units/min 3 min after the reduction of external Na^+ to 1/10 (9 mM), from which a maximum HCO_3^- efflux rate of 7.6 mM/min was calculated. In three other experiments of this kind similar accelerations of the initial intracellular acidification were measured 2–4 min after reducing the external Na^+ concentration.

Second, when the external Na^+ concentration was reduced for 10 min or longer, time enough for the intracellular Na^+ to decrease to 1–2 mM (not shown), the rate of intracellular acidification upon lowering the external pH was not altered or even

reduced in the presence of CO_2/HCO_3^- (Fig. 6 *B*). Indeed, the mean maximum rate of intracellular acidification was similar in CO_2/HCO_3^--free saline and in low external Na^+ with or without CO_2/HCO_3^-. The rates of intracellular acidification in pH_o 7.0 were 0.02, 0.017, and 0.016 pH units/min, respectively. This type of experiment indicated that the HCO_3^--independent intracellular acidification was unaffected by changing the Na^+ gradient.

At a very low intracellular Na^+, the HCO_3^--dependent rate of acidification was greatly reduced. When the intracellular Na^+ was depleted, the presence of CO_2/HCO_3^- did not alter the rate of intracellular acidification, suggesting that the HCO_3^- efflux as such was inhibited.

This experiment was repeated with slightly different protocols; they all indicated a much reduced intracellular acidification, when the external Na^+ concentration had been lowered for 10–30 min.

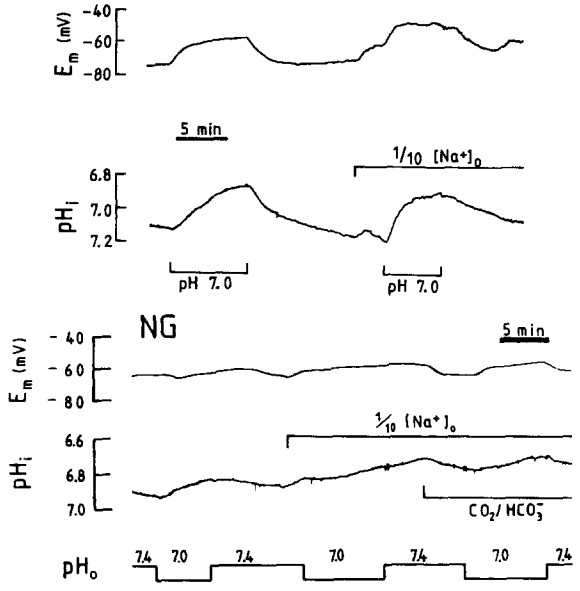

FIGURE 6. The role of the Na^+ gradient across the glial membrane for the rate of intracellular acidification. (*A*) After a control in normal Na^+, the external Na^+ concentration was reduced to 1/10 normal (9 mM) 3 min before exposing the preparation again to pH 7.0. (*B*) Repeated exposures to pH 7.0 in CO_2/HCO_3^--free saline, normal Na^+, and 1/10 normal Na^+ concentration; after 18 min in 1/10 normal Na^+ lowering the external pH to 7.0 did not alter the rate of intracellular acidification even in the presence of CO_2/HCO_3^-.

The reduction of the external Na^+ concentration caused a membrane depolarization which was amplified by reducing the external pH within a few min of the Na^+ reduction. After a longer exposure to low external Na^+, however, lowering external pH not only produced a greatly slowed intracellular acidification, but also a much reduced membrane depolarization. This is in line with the suggestion that the membrane depolarization accompanying the rapid intracellular acidification was due to Na^+-dependent electrogenic HCO_3^- efflux.

Effect of Amiloride

The experiments described above showed that the rate of intracellular acidification was dependent on the Na^+ gradient. To exclude that the effects might be produced by changing the activity of the Na^+-H^+ exchanger, which also runs on the Na^+

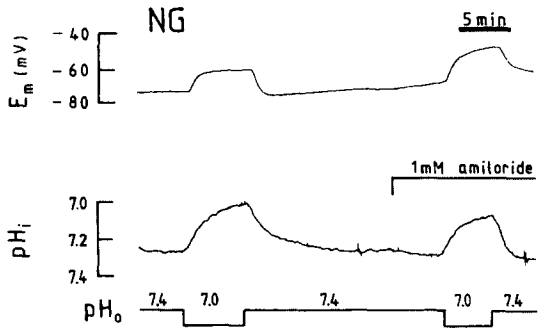

FIGURE 7. The effect of amiloride on the rate of intracellular acidification after the reduction of external pH.

gradient and which was shown to exist in the glial cell membrane (Deitmer and Schlue, 1987), amiloride was applied before and during the change in external pH (Fig. 7). There was no effect of amiloride on the rate of intracellular acidification in the presence of CO_2/HCO_3^- in these experiments (n = 4).

Measurements of the Intracellular Na Activity

The aNa_i of the neuropile glial cells was 5.2 ± 1.0 mM (n = 8) in CO_2/HCO_3^--free saline. After introduction of CO_2/HCO_3^-, aNa_i increased by several millimoles/liter (Fig. 8). Often there was a transient peak before aNa_i settled near 7 mM; on average, aNa_i was 7.4 ± 2.2 mM (n = 8) in a saline containing 5% CO_2/24 mM HCO_3^-. The equilibrium potentials for Na^+ were +65 mV in the absence and +56 mV in the presence of 5% CO_2/24 mM HCO_3^- (Table I). With the membrane potentials of −65 and −74 mV in these two salines, the electrochemical gradient for Na^+ remained virtually constant, being 131 and 130 mV, respectively.

Reducing the pH_o from 7.4 to 7.0 produced a small increase of aNa_i in the CO_2/HCO_3^--free saline (Fig. 8), which could be due to some stimulation of Na^+-H^+ exchange during the induced intracellular acidification. In the presence of CO_2/HCO_3^-, however, aNa_i decreased significantly in the lowered external HCO_3^- concentration (pH_o 7.0), on average by 2.0 ± 1.1 mM (n = 5). Thus, the aNa_i at lowered external pH (7.0) was comparable to the value measured in the absence of CO_2/HCO_3^- at pH_o 7.4 (Table I). After restoring pH_o to 7.4, aNa_i increased rapidly to its initial value.

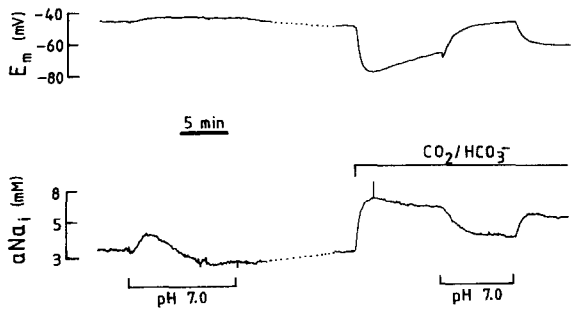

FIGURE 8. Changes in the aNa_i (*lower trace*) and the membrane potential (*upper trace*) after the reduction of external pH from 7.4 to 7.0 in the absence and in the presence of 5% CO_2/HCO_3^-.

These experiments indicate that the HCO_3^- efflux was accompanied by a reduction of intracellular Na^+. On the other hand, restoration of the external pH, which led to a rapid intracellular HCO_3^--dependent alkalinization, was accompanied by an increase in aNa_i. The pH_o-dependent changes in aNa_i strongly suggest that the fluxes of HCO_3^- across the cell membrane were coupled to Na^+ movements in the same direction.

Acid/Base Flux across the Neuronal Cell Membrane

For comparison, some of the experiments described above were also performed while measuring pH_i and membrane potential from identified neurons in intact leech ganglia, such as P(ressure) cells and Retzius cells. The intracellular acidification in these neurons after a step change of external pH from 7.4 to 7.0 was also faster in the presence than in the absence of CO_2/HCO_3^- (Fig. 9). The mean maximum rate of intracellular acidification in Retzius neurons was 0.01 ± 0.004 pH units/min (n = 12) in CO_2/HCO_3^--free saline, and 0.022 ± 0.01 pH units/min (n = 14) in the presence of

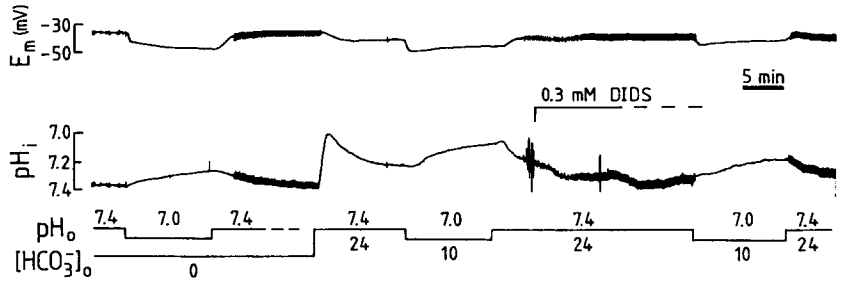

FIGURE 9. pH_i and membrane potential measurement in a Retzius neuron. The external pH was repeatedly lowered from 7.4 to 7.0 in the absence and presence of 5% CO_2/HCO_3^- and after the addition of 0.3 mM DIDS.

5% CO_2/10 mM HCO_3^-. This indicated acid/base fluxes of 0.2 and 1.3 mM/min, respectively (Table II). The rate of acidification in the presence of CO_2/HCO_3^- was somewhat reduced by DIDS (Fig. 9), but was unaffected by amiloride (not shown). This suggested that the transport of acid/base equivalents was due in part to HCO_3^- fluxes across the neuronal membrane.

The aNa_i of Retzius neurons increased upon lowering the external pH both in the absence and in the presence of CO_2/HCO_3^-. Lowering the external Na^+ concentration had little or no effect on the rate of intracellular acidification. It is therefore concluded that the Na^+ gradient was not involved in driving acid equivalents across the neuronal membrane into the cells.

In Retzius neurons the membrane rapidly hyperpolarized by 7–10 mV upon lowering pH to 7.0, both in the presence and absence of CO_2/HCO_3^-. This membrane hyperpolarization occurred even before any change in pH_i was observed (Fig. 9). The spontaneously firing neurons became silent and only resumed their electrical activity after restoring the external pH to 7.4. It seems that Retzius neurons are very sensitive to extracellular pH changes, a phenomenon reported for a variety of neurons

(Eyzaguirre and Koyano, 1965; Fukuda and Loeschcke, 1977; Jarolimek et al., 1990). This indicates the importance of extracellular pH changes for neuronal activity, and the demand for active regulation also of the extracellular pH.

DISCUSSION

This study shows that, when pH_o is decreased, the rate of intracellular acidification in leech neuropile glial cells is dependent on the presence of CO_2/HCO_3^-. It is suggested that this acidification is produced by a Na^+-coupled HCO_3^- efflux, due to an outwardly directed electrogenic Na^+-HCO_3^- cotransport. The main evidence is the following: (*a*) The rate of intracellular acidification and the membrane depolarization were dependent on the external HCO_3^- concentration and pH_i (and hence on the intracellular HCO_3^- concentration). (*b*) The stilbene DIDS blocked the HCO_3^--dependent rate of intracellular acidification. (*c*) Reduction of the external Na^+ concentration changed the acid/base flux across the membrane in a time-dependent fashion as expected for a transport process coupled to Na^+ efflux. (*d*) The aNa_i decreased during the intracellular acidification in the presence, but increased in the absence of CO_2/HCO_3^-. (*e*) The rate of intracellular acidification was unaffected by the removal of external Cl^-, or by the addition of amiloride, an inhibitor of the Na^+-H^+ exchange. The results are consistent with the conclusion that the electrogenic Na^+-HCO_3^- cotransport, which was previously described as an inwardly directed carrier in leech glial cells (Deitmer and Schlue, 1989; Deitmer and Szatkowski, 1990) can be reversed under appropriate thermodynamic conditions.

Factors Determining the Mode of Na^+-HCO_3^- Cotransport

The reversibility of this acid/base transport system appears to be a key characteristic with respect to the role of glial cells in pH regulation. The direction of acid/base transport across the glial membrane depends on the thermodynamic conditions. These are given by the gradients of H^+/HCO_3^- and Na^+, the stoichiometry of the Na^+-HCO_3^- cotransporter, and the membrane potential. For the inwardly directed Na^+-HCO_3^- cotransport, a stoichiometry of 1 Na^+:2 HCO_3^- was determined (Deitmer and Schlue, 1989). At steady-state values of intra- and extracellular Na^+ and HCO_3^-, this gave an equilibrium potential for Na^+-HCO_3^- cotransport of −90 mV. In the present study with the somewhat modified salines (lower NaCl and higher CO_2/HCO_3^- concentration than in our previous studies; see Deitmer and Schlue, 1987, 1989; Deitmer and Szatkowski, 1990) a stoichiometry of 1:2 would indicate an equilibrium potential of −74 mV, a value identical to the mean resting potential of the glial cells in the presence of 5% CO_2/24 mM HCO_3^-. This suggests that at steady state, the Na^+-HCO_3^- cotransport is at equilibrium, where no net transport of acid/base equivalents occurs across the glial membrane.

It should be mentioned that in this preparation no distinction has been defined between a transport of two HCO_3^- or one CO_3^{2-} coupled to one Na^+ in the cotransporter. This may depend on the stoichiometry of the cotransporter, 1:2 or 1:3, as recently discussed by Boron and Boulpaep (1989).

When the external pH is reduced from 7.4 to 7.0, which corresponds to a decrease in the external HCO_3^- concentration from 24 to 10 mM, the Na^+-HCO_3^- cotrans-

porter is far from equilibrium; its equilibrium potential, assuming a constant stoichiometry of 1:2, would shift from −74 to −30 mV. This would activate Na^+-HCO_3^- cotransport in the outwardly directed mode to approach a new equilibrium. The glial membrane depolarized during this change in external pH by a mean of 20 mV from −74 to −54 mV, and pH_i decreased by ~0.2 pH units.

At pH_o 7.0, the pH_i approached a value near 7.05, from which an equilibrium potential of the Na^+-HCO_3^- cotransporter of −60 mV was calculated. This is fairly close to the mean membrane potential value in this saline (−54 mV). It appears, therefore, that the membrane potential and the equilibrium potential of the Na^+-HCO_3^-cotransporter come close to each other in salines of various pH and HCO_3^- concentrations.

Assuming a stoichiometry of 1 Na^+:3 HCO_3^-, the equilibrium of the Na^+-HCO_3^- cotransporter would be −41 mV at steady-state conditions in 5% CO_2/24 mM HCO_3^- (pH_o 7.4). This would drive Na^+ and HCO_3^- out of the cell at the resting potential of −74 mV. Since there is no apparent mechanism other than the Na^+-HCO_3^- cotransporter to maintain the glial cytoplasm alkaline (7.25) with respect to the H^+ equilibrium (which would be 6.13 at these conditions) a stoichiometry of 1:3 or more seems unlikely. Furthermore, returning from pH_o 7.0 to 7.4 would not hyperpolarize the glial membrane and alkalinize the glial cytoplasm as much as it actually does if the stoichiometry was more than 1:2. The present experiments therefore suggest a stoichiometry of 1 Na^+:2 HCO_3^- for both directions of the cotransporter.

It is also interesting to compare the equilibrium potentials and electrochemical gradients of the ions involved, Na^+ and HCO_3^-, in the different salines (Table I). While the Na^+ gradient remains virtually the same with and without CO_2/HCO_3^-, the H^+/HCO_3^- gradient increased from 40 to 65 mV in the presence of 5% CO_2/24 mM HCO_3^-. This was suggested to be due to the activity of the Na^+-HCO_3^- cotransporter, which is responsible for the alkaline pH_i in this saline (Deitmer and Schlue, 1987, 1989).

Occurrence of Electrogenic Na^+-HCO_3^- Cotransport in Glial Cells

Evidence for a DIDS-sensitive Na^+-HCO_3^- cotransport has been presented from a variety of different types of glial cells, such as glial cells from amphibian optic nerve (Astion and Orkand, 1988) and cultured mouse oligodendrocytes (Kettenmann and Schlue, 1988), as well as in frog retinal pigment epithelium, which is part of the blood–retinal barrier and may be regarded as glial-like tissue (Hughes, Adorante, Miller, and Lin, 1989; LaCour, 1989). The stoichiometry in all these glial cells was reported to be greater than 1. In frog retinal pigment epithelium (Hughes et al., 1989) and in leech neuropile glial cells (Deitmer and Schlue, 1989) a stoichiometry of 1:2 was suggested. In kidney epithelia, a stoichiometry of 1:2 (Alpern, 1985; Jentsch, Schwartz, Schill, Langner, Lepple, Keller, and Wiederholt, 1986) and of 1:3 (Yoshitomi, Burckhardt, and Frömter, 1985; Lopes, Siebens, Giebisch, and Boron, 1987; Soleimani, Grassl, and Aronson, 1987) has been reported. It is unclear whether this cotransporter occurs with different stoichiometries or different binding sites for CO_3^{2-} and/or HCO_3^- (Boron and Boulpaep, 1989).

In frog retinal pigment epithelium, a reversal of the membrane response, pre-

sumed to be due to electrogenic Na^+-HCO_3^- cotransport, occurred at −114 mV (Hughes et al., 1989). While the normal direction of the cotransport is carrying Na^+, HCO_3^-, and negative charge into the cells, this direction can be reversed by strong membrane hyperpolarization or by the removal of Na^+ and/or HCO_3^-. Although the equilibrium potential of the Na^+-HCO_3^- cotransporter appears more negative in frog pigment epithelium than in leech neuropile glial cells, there seems to be a remarkable similarity in the properties of the electrogenic Na^+-HCO_3^- cotransport in these two types of cells.

Functional Significance of Base Secretion from Glial Cells

If glial cells were to play a role in the regulation of extracellular (interstitial) pH, they should be able to transport acid/base equivalents across their membranes in both directions. Their ability to remove base equivalents effectively, and thereby to damp extracellular alkalosis, has been suggested to occur mainly by an inwardly directed, electrogenic Na^+-HCO_3^- cotransporter (Deitmer and Schlue, 1987, 1989). This process alkalinizes the glial cell and, due to accumulation of HCO_3^-, would increase their intracellular buffering power.

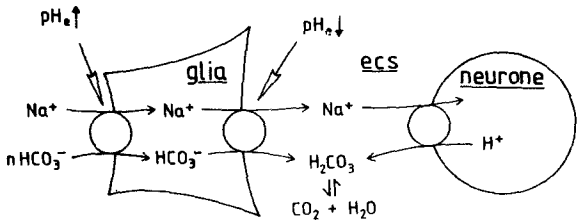

FIGURE 10. Schematic diagram of the inwardly and outwardly directed electrogenic (n = 2) Na^+-HCO_3^- cotransport across the glial cell membrane, stimulated by raising or lowering external pH (pH_e ↑, pH_e ↓). The outwardly directed cotransport would provide the base equivalents to neutralize protons extruded by the neurons via Na^+-H^+ exchange into the extracellular space (*ecs*). Thus, extracellular alkalosis and acidosis could be counteracted by uptake and secretion of HCO_3^- by glial cells. See text for further description.

The diagram in Fig. 10 summarizes the impact of inwardly and outwardly directed electrogenic Na^+-HCO_3^- cotransport across the glial membrane for the regulation of intraglial and extracellular pH. We have shown that the inward movement of HCO_3^- is stimulated not only by increasing the external HCO_3^- concentration (and/or decreasing external pH), but also by membrane depolarization, caused, for example, by a rise in extracellular K^+ or by other substances that depolarize the membrane (Deitmer and Szatkowski, 1990).

In this paper it is shown that after extracellular acidosis the electrogenic Na^+-HCO_3^- cotransporter in the glial membrane can be reversed. Base equivalents are secreted due to HCO_3^- efflux into the extracellular space. This would tend to reduce a given extracellular acid load, as may occur during neuronal activity (cf. Syková, Svoboda, Chvátal, and Jendelová, 1988; Chesler, 1990). Due to the efflux of HCO_3^- into the extracellular spaces, the buffering power in these spaces increases (Deitmer, 1991). Ammonium-induced extracellular pH transients were significantly

reduced in the presence of CO_2/HCO_3^-, indicating an increased interstitial buffer capacity.

The pH regulation by leech neurons mainly involves Na^+-H^+ exchange (Schlue and Thomas, 1985; Deitmer and Schlue, 1987). The extruded H ions from neurons would then be neutralized in the extracellular space by HCO_3 ions secreted by the glial cells (Fig. 10). Neurons take up acid or secrete base much less rapidly than glial cells, as indicated by the relatively slow intracellular acidification of Retzius neurons. Although the rate of this acidification was also higher in the presence of CO_2/HCO_3^-, it was still about three times lower than that in glial cells. The rate of intracellular acidification in neurons, in contrast to glial cells, was not affected by the Na^+ gradient. This is in accordance with the finding that neurons do not possess a Na^+-HCO_3^- cotransport (Deitmer and Schlue, 1987, 1989). On the other hand, Cl^--HCO_3^- exchange may be involved in transporting acid/base equivalents across the neuronal membrane under these conditions.

In conclusion, the electrogenic Na^+-HCO_3^- cotransporter of glial cells can operate in both directions, and may, depending on the thermodynamic conditions, effectively secrete or take up acid/base equivalents. Due to the membrane potential dependence of the Na^+-HCO_3^- cotransport (Deitmer and Szatkowski, 1990), modulators of the glial membrane potential, such as extracellular K^+ or neurotransmitters, could stimulate or inhibit this cotransport, and/or determine the direction of acid/base transport into or out of glial cells. This may be a key mechanism to damp ("muffle") extracellular pH changes and to contribute to the overall H^+ homeostasis in the nervous system.

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