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Further Studies of Electrogenic Na^+/HCO_3^- Cotransport in Glial Cells of *Necturus* Optic Nerve: Regulation of pH_i

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ABSTRACT In the presence of Ba^{++}, an increase in the bath HCO_3^- at constant CO_2 (i.e., variable bath pH) produced a hyperpolarization. The hyperpolarizing effect of adding HCO_3^-/CO_2 at constant bath pH was not significantly affected by the presence of 50 μmol/l strophanthidin. In the absence of Ba^{++}, addition of HCO_3^-/CO_2 at constant bath pH produced a Na^+-dependent hyperpolarization. Therefore, CO_2 movements, electrogenic Na^+/K^+ pump activity and changes in Ba^{++} binding do not contribute significantly to the hyperpolarization induced by HCO_3^-. These results along with the results of previous studies (Astion et al: J Gen Physiol 93:731, 1989) strongly suggest that the hyperpolarization induced by the addition of HCO_3^- is due to an electrogenic Na^+/HCO_3^- cotransporter, which transports Na^+, HCO_3^- (or its equivalent), and net negative charge across the glial membrane.

To study the role of electrogenic Na^+/HCO_3^- cotransport in the regulation of pHi in glial cells, we used intracellular double-barreled, pH-sensitive microelectrodes. At a bath pH of 7.5, the mean initial intracellular pH (pH_i) was 7.32 (SD 0.03, n = 6) in HEPES-buffered Ringer's solution and 7.39 (SD 0.1, n = 6) in HCO_3^-/CO_2 buffered solution. These values for pH_i are more than 1.2 pH units alkaline to the pH_i predicted from a passive distribution of protons; thus, these cells actively regulate pH_i. Superfusion and withdrawal of 15 mmol/l NH_4^+ induced an acidification of 0.2 to 0.3 pH units, which recovered toward the original steady-state pH_i. Recovery from acidification was stimulated by adding HCO_3^-/CO_2 at constant pH. In HCO_3^-/CO_2-buffered solutions, the recovery was Na^+-dependent, inhibited by 4-acetamido-4′- isothiocyanato-stilbene-2,2′-disulfonic acid (SITS), and associated with a hyperpolarization of the membrane. Thus it appears that the electrogenic Na^+/HCO_3^- cotransporter helps maintain the relatively alkaline pH_i of glial cells and also contributes to the ability of glial cells to buffer changes in pH in the neuronal microenvironment.

INTRODUCTION

In a variety of cells of epithelial origin, an electrogenic Na^+/HCO_3^- cotransporter has been described in which Na^+, HCO_3^- (or its equivalent), and net negative charge move in the same direction across the cell membrane (Boron and Boulpaep, 1983b; for review see Boron, 1986). The hypothesis of electrogenic Na^+/HCO_3^- cotransport predicts that addition of external HCO_3^- should cause a hyperpolarization that is Na^+-dependent, sensitive to disulfonic stilbenes, e.g., 4-acetamido-4′- isothiocyanato-stilbene-2,2′-disulfonic acid (SITS), and independent of Cl^-. The hyperpolarization should

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TABLE 1. Composition of the most commonly used Ringer's solutions (in mmol/l)

	Solution[a]			
	1 (NR, HEPES)	2 (2 Ba^{++}, HEPES)	3 (NR, HCO_3^-)	4 (2 Ba^{++}, HCO_3^-)
NaCl	110	110	100	100
KCl	3	3	3	3
$CaCl_2$	2	0	2	0
$BaCl_2$	0	2	0	2
HEPES	5	5	0	0
$NaHCO_3^-$	0	0	12.5	12.5
CO_2	RA	RA	2.3%	2.3%
O_2	RA	RA	97.7%	97.7%
pH[b]	7.5	7.5	7.5	7.5

[a]NR, normal Ringer's solution; RA, open to room air but not bubbled.
[b]Solutions 1 and 2 were titrated with 1 M NaOH to their final pH.

not be due solely to other electrogenic processes like the Na^+/K^+ ATPase or pH-sensitive conductances. In previous studies, we have shown that the addition of HCO_3^-/CO_2 at constant bath pH induces a hyperpolarization of the glial membrane, which is Na^+-dependent, SITS-sensitive (Astion et al., 1989b), and Cl^--independent (Astion et al., 1987). All the results were obtained in the presence of 2–5 mmol/l Ba^{++}, which reduces the glial K^+ conductance (Astion et al., 1989b). With a decreased glial K^+ conductance, the HCO_3^--induced hyperpolarization is larger and is thus easier to investigate (Astion et al., 1987). The data are consistent with the existence of an electrogenic Na^+/HCO_3^- cotransporter in the glial membrane. Such a result is of special interest because this process, found in glia but not in neurons, could contribute to a role of glial cells in buffering extracellular pH in the nervous system.

In the previous studies we did not investigate the possibility that CO_2 movements, Na^+/K^+ ATPase activity, and/or changes in Ba^{++} binding might account for the hyperpolarizing effects of adding HCO_3^-/CO_2. Movements of CO_2 are of concern because they lead to rapid changes in pH_i, which may affect pH-sensitive conductances. In the first part of this work, we explore these possibilities with recordings of membrane potential. In the second part, glial cells were impaled with double-barreled, pH-sensitive microelectrodes to determine the possible role of electrogenic Na^+/HCO_3^- cotransport in pH_i regulation in glial cells.

MATERIALS AND METHODS

Experimental Preparation

Adult *Necturus maculosus* were maintained in tap water containing a few drops of 1% methylene blue at 2–4°C. After decapitation, the optic nerve was exposed through a window in the roof of the mouth. The nerve was desheathed in situ and transferred to a 0.1 ml chamber containing a normal Ringer's solution (solution 1 or 3, Table 1). The nerve was continuously superfused at 15 to 30 bath volumes per minute via two six way taps. Solutions that were equilibrated with room air were delivered to the tap by gravity through polyethylene tubing. Gas impermeable tubing (Norton Performance Plastics, Wayne, NJ) was used to deliver solutions containing bubbled gasses. Before a solution containing bubbled gasses was passed into the chamber, the line was bled with fresh solution.

Solutions

The composition of the most commonly used Ringer's solutions are given in Table 1. Na^+-free solutions were made by replacing NaCl with N-methyl-D-glucamine-chloride (NMDG-Cl); NMDG-Cl was prepared by neutralizing N-methyl-D-glucamine ($NMDG^+$) with HCl. $NaHCO_3^-$ was replaced by choline-HCO_3^-. HEPES was neutralized with N-methyl-D-glucamine. $NMDG^+$ and choline-HCO_3^- were purchased from Sigma (St. Louis, MO).

For NH_4^+-containing solutions, NH_4Cl replaced NaCl and the NH_4Cl was added as a powder close to the time of the experiment. Solutions containing SITS were kept shielded from light. SITS and strophanthidin were purchased from Sigma.

Microelectrodes

Conventional microelectrodes were made from single-barreled, thick-walled, borosilicate glass capillaries that contained a filament (WPI, New Haven, CT). After being pulled, (Kopf model 700B vertical puller; Tujunga, CA) the electrodes were filled with 3 M KCl.

Double-barreled pH-sensitive microelectrodes were constructed from borosilicate theta capillaries (WPI). The freshly pulled electrodes were silanized using a modification of the method of Munoz et al. (1983). The capillary was mounted horizontally on a micromanipulator, and a syringe needle was placed about half-way down each barrel. Argon was passed continuously through one barrel (reference barrel) and vapors of trimethyldimethylaminosilane (Fluka, Ronkonkoma, NY) were passed through the other (sensor barrel).

After 1 min of passing silane vapors, the electrode was placed in the center of a heating coil (250°C) where it remained for 5 min. The silanized barrel was filled with the pH sensor of Ammann et al. (1981; Fluka 82500 or 95297). After filling the tip with the sensor, the sensor barrel was backfilled with a solution containing (in mmol/l): 40 KH_2PO_4, 23 NaOH, 15 NaCl, pH 7.0. The reference barrel was filled with 3 M KCl.

The pH-sensitive electrodes were calibrated in normal Ringer's solutions of pH 7.5 (solution 1) and 6.8. The pH 6.8 solution had the identical ionic composition to the pH 7.5 solution except that it contained PIPES (pK_a = 6.8) instead of HEPES (pK_a = 7.5). The electrodes were tested for their response to CO_2 by switching back and forth from a HEPES-buffered solution (solution 1) to a HCO_3^-/CO_2-buffered solution (solution 3) at a constant pH of 7.5. If there was a response to CO_2 the electrode was rejected. Double-barreled electrodes were accepted that had slopes within 7 mV of the Nernstian slope (58 mV/pH unit).

Recording Arrangement

Experiments were performed in a Faraday cage. After an impalement was made, the front of the cage was covered with Velostat (3M Company, Minneapolis, MN). The bath was grounded via a freshly coated Ag/AgCl wire that was connected to the bath through a 3 M KCl agar bridge (0.25 mm diameter) placed near the suction outflow. Each barrel of the pH-sensitive microelectrode was connected with Ag/AgCl wires to the input of a Burr Brown OPA 128LM operational amplifier. The output of the reference barrel was subtracted from the sensor barrel signal to produce the ion signal. The ion signal and the reference output were transferred to a Kipp and Zonen BD 41 chart recorder (Delft, Holland). Single-barrel KCl electrodes were connected to an electrometer (WPI, M4A). The ion and reference signals were passed through a modified Sony PCM-501ES digital audio processor (Unitrade Inc., Philadelphia, PA) before being stored on a video cassette recorder (Sony SL2700, Beta HI-FI). The data recorded in this manner were available for reproduction and analysis at a later time (Bezanilla, 1985).

RESULTS

Conventional Microelectrode Studies: Further Analysis of the Hyperpolarization Induced by HCO_3^-

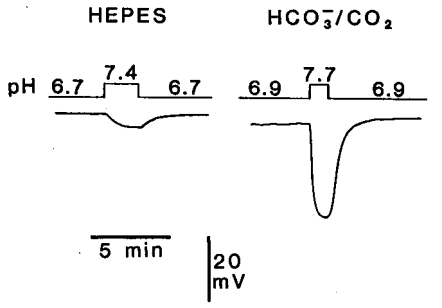
There are at least two factors that may contribute to the hyperpolarization of the glial membrane produced by adding HCO_3^-/CO_2 at constant pH in the presence of Ba^{++} (Astion et al., 1987, 1989b). One factor is an influx of HCO_3^- resulting from a change in its equilibrium potential, and the other is the rapid influx of CO_2. Inside the cell, CO_2 is hydrated to H_2CO_3 and dissociates into H^+ and HCO_3^-, thereby causing an acidification (Roos and Boron, 1981). This acidification may alter pH-sensitive conductances (e.g., Keller et al., 1986) and decrease Ba^{++} binding by titrating barium binding sites in the K^+-channel. The effect of CO_2 influx was eliminated by raising bath [HCO_3^-] at constant CO_2 (i.e., variable pH); this protocol is associated with an intracellular alkalinization whose magnitude depends on the extent of membrane transport processes for HCO_3^- (Boron and Boulpaep, 1983b). The results of a typical experiment are shown in Fig. 1. The record on the right side shows that increasing the bath [HCO_3^-] from 7.1 to 40 mmol/l at a constant CO_2 of 5% (i.e., changing the bath pH from 6.9 to 7.7) induces a reversible hyperpolarization of about 30 mV. The record on the left is a control from the same impalement that shows the much smaller hyperpolarization produced by raising bath pH from 6.7 to 7.4 in the absence of HCO_3^-/CO_2. The results strongly suggest that HCO_3^- hyperpolarizes glial membranes by a mechanism that does not require CO_2 movements.

Glial cells of *Necturus* optic nerve possess a strophanthidin-sensitive, hyperpolarizing, Na^+/K^+ ATPase (Tang et al., 1980). Addition of HCO_3^-/CO_2 might cause an increase in $[Na^+]_i$ (e.g., Thomas, 1977) that might activate this electrogenic transport mechanism. Fig. 2 shows two records from a single impalement that are concerned with this issue. HCO_3^-/CO_2 (12.5 mmol/l HCO_3^-, 2.3% CO_2) was added and removed at constant bath pH in the presence (upper record) or absence (lower record) of 50 μmol/l strophanthidin. The addition of strophanthidin caused a reversible 3 mV depolarization, presumably due to inhibition of the Na^+/K^+ ATPase. The initial HCO_3^--induced hyperpolarization was unaffected by strophanthidin, suggesting that the electrogenic Na^+/K^+ ATPase does not contribute.

The experiments above, as well as previous studies (Astion et al., 1987, 1989b), were performed in the presence of Ba^{++}, a well-known K^+-channel blocker (e.g., Armstrong et al., 1982). Addition of 2–5 mmol/l Ba^{++} depolarizes glial cells of *Necturus* optic nerve by 25–50 mV and decreases the K^+ conductance (Astion et al., 1989b). We were concerned that the initial acidification that follows addition of HCO_3^-/CO_2 (see results below) might decrease the binding of Ba^{++} in K^+-channels, and thereby cause a hyperpolarization. To examine the possibility that a change in Ba^{++} binding was responsible for the effects of HCO_3^-, we did experiments in the absence of Ba^{++}. Fig. 3 shows that in the absence of Ba^{++}, addition of 26 mmol/l HCO_3^-/5% CO_2 at constant bath pH caused a hyperpolarization of 5 mV that was Na^+-dependent, indicating that the effects of HCO_3^- are not due solely to changes in Ba^{++} binding.

Studies With pH-Sensitive Microelectrodes

To determine the relation between pH_i and the electrogenic Na^+/HCO_3^- cotransporter, we impaled the glial cells with double-barreled, pH-sensitive microelectrodes. Stable, long-lasting impalements were difficult



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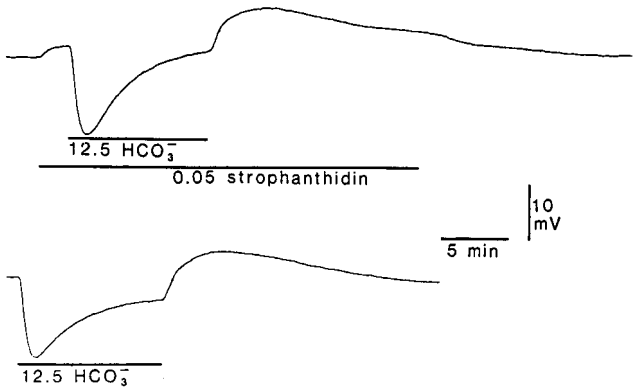

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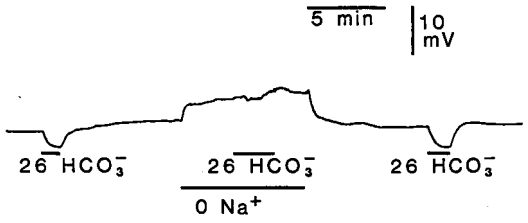

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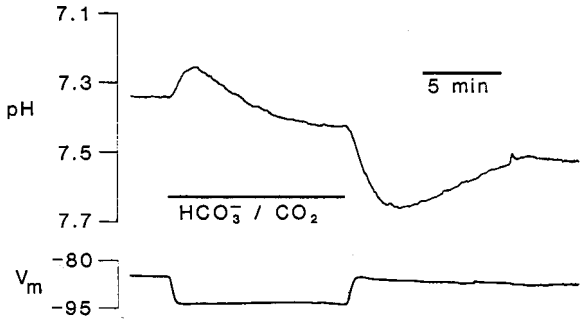

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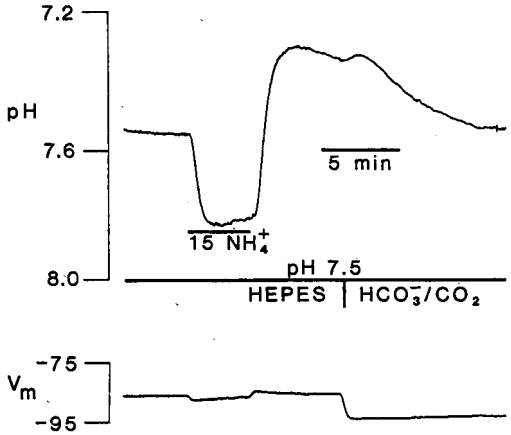

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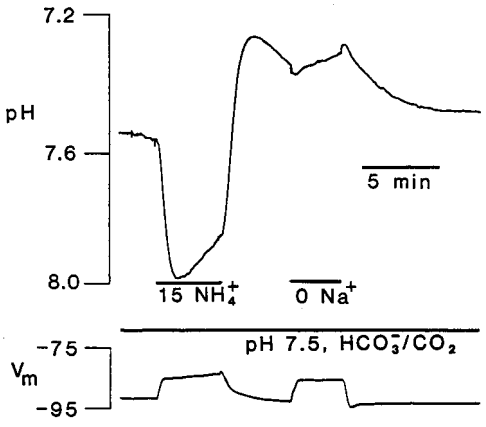

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Figs. 1–6.

to obtain. For an impalement to be considered valid, the pH_i and the resting potential had to be stable for at least 5 min and the resting potential had to be more negative than −70 mV.

At a bath pH of 7.5, the mean initial pH_i was 7.32 (SD = 0.03, n = 6) in HEPES-buffered Ringer's solution (solution 1) and 7.39 (SD = 0.1, n = 6) in Ringer's solution buffered with HCO_3^-/CO_2 (solution 3). The means in the two different buffers were not significantly different ($P > 0.1$, t-test, df = 10). Fig. 4 shows the change in pH_i and membrane potential when the 5 mmol/l HEPES buffer was replaced by 12.5 mmol/l HCO_3^-, 2.3% CO_2 at constant pH (7.5). Addition of HCO_3^-/CO_2 hyperpolarized the cell and produced a transient acidification. Presumably, the acidification is due to the rapid influx of CO_2, hydration of CO_2 to form carbonic acid, and dissociation of carbonic acid into H^+ and HCO_3^- (Roos and Boron, 1981). The hyperpolarization is Na^+-dependent (Fig. 3) and is probably due to influx of Na^+, HCO_3^-, and net negative charge via the electrogenic Na^+/HCO_3^- cotransporter. In about 50% of the recordings, including that in Fig. 4, the initial HCO_3^--induced hyperpolarization was maintained in the continued presence of HCO_3^-/CO_2. In other cells, the initial hyperpolarization was followed by a recovery toward pre-HCO_3^- resting potential (Astion et al., 1987). Removal of HCO_3^-/CO_2 caused a depolarization and a transient alkalinization, presumably due to the efflux of CO_2.

The pH_i predicted from a passive distribution of protons at a resting potential of −80 mV and a pH_o of 7.5 is 6.1. Thus, in the presence of either HEPES or HCO_3^-/CO_2, glial cells actively buffer acid. We wanted to know if electrogenic Na^+/HCO_3^- cotransport tends to drive the pH_i to the steady-state value of 7.4 (in HCO_3^-/CO_2). If this is true, then the recovery from an intracellular acidification should be Na^+-dependent, HCO_3^--dependent, SITS-sensitive, and associated with an inward movement of negative charge. The results we now present support the hypothesis that, in HCO_3^-/CO_2, electrogenic Na^+/HCO_3^- drives the pH_i to near the steady state pH_i.

The NH_4^+ withdrawal technique (for review and discussion, see Roos and Boron, 1981) was used to acidify the glial cells (Figs. 5–8). Application of NH_4^+ for a few minutes leads to an initial alkalinization, caused by movement of NH_3 into the cell, followed by a slowing and eventual reversal of the alkalinization as NH_4^+ enters the cell down its electrochemical gradient. Removal of NH_4^+ from the bath leads to a pH_i that is more acid than that observed before the addition of NH_4^+. The acidification results from the unequal driving forces for NH_4^+ entry and NH_4^+ exit. With a negative resting potential, the driving force for NH_4^+ entry when external NH_4^+ is applied is much greater than the driving force for NH_4^+ exit when external NH_4^+ is removed. Thus, more NH_4^+ enters than exits the cells. The excess intracellular NH_4^+ dissociates into NH_3 and H^+, thereby producing the acidification. For the experiments described below 15 mmol/l NH_4^+ was added for approximately 4 min. Upon withdrawal of the 15 mmol/l NH_4^+, the glial cells acidified by 0.2–0.3 pH unit after which the pH_i recovered toward the original steady state.

Fig. 5 shows an experiment in which the dependence of acid extrusion on HCO_3^- was examined. The cells were acidified in the nominal absence of HCO_3^- by addition and withdrawal of 15 mmol/l NH_4^+. To quantitate the effect of the treatment we measured the maximum rate of recovery after the peak of the acidification and compared it to the maximum rate of recovery after adding HCO_3^-. As the slope of the recovery decreases with time

Fig. 1. Effect of raising bath pH in the presence and absence of HCO_3^-/CO_2. Records from the same impalement; experiment done in the presence of 2 mmol/l Ba^{++}. In the left record the bath pH was raised from 6.7 to 7.4 in the nominal absence of HCO_3^-/CO_2. The composition of the solution was (in mmol/l): 110 NaCl, 3 KCl, 2 $BaCl_2$, 5 HEPES (pH to 6.7 or 7.4 with NaOH). The break in the record is 25 min during which time the nerve was superfused with a solution containing 103 NaCl, 3 KCl, 2 $BaCl_2$, 7.1 $NaHCO_3^-$ 5% CO_2, pH 6.9. In the right record the pH was raised from 6.9 to 7.7 by raising the HCO_3^- (HCO_3^- replaced Cl^-) from 7.1 to 40 mmol/l at constant CO_2. This caused a hyperpolarization that was much larger than that observed in the absence of HCO_3^-. Initial V_m = −44 mV and −47 mV for left and right records.

Fig. 2. The membrane potential changes induced by adding HCO_3^- at constant bath pH are not significantly affected by strophanthidin (0.05 mmol/l). Upper and lower records from the same impalement. The initial (upper record) Ringer's solution contained (in mmol/l): 110 NaCl, 3 KCl, 2 $BaCl_2$, 0.4 Na_2HPO_4, 0.1 NaH_2PO_4, 10 HEPES titrated with 5 NaOH to pH 7.5. In the first part of the record, 0.05 mmol/l strophanthidin was added; this induced a small, reversible, depolarization. Next, 12.5 mmol/l HCO_3^- was added at constant bath pH (7.5) in the presence of strophanthidin. HCO_3^--containing solutions were bubbled with 2.3% CO_2; HCO_3^- replaced Cl^-. The lower record is a strophanthidin-free control from the same impalement. The lower record begins 3 min after the end of the upper record. Initial V_m = −52 mV and −50 mV for the upper and lower records.

Fig. 3. In the absence of Ba^{++}, addition of HCO_3^- at constant bath pH (7.5) induces a hyperpolarization that is Na^+-dependent. The middle part of the record shows the effect of adding 26 mmol/l HCO_3^- when the cell had been bathing in a solution in which Na^+ had been removed. The left and right sections of the continuous record are controls showing the effect of adding HCO_3^- in the presence of Na^+. Initial V_m = −88 mV. The nominally HCO_3^--free Ringer's solution was solution 1. The HCO_3^-/CO_2 buffered Ringer's solution contained (in mmol/l): 89 NaCl, 3 KCl, 2 $CaCl_2$, 26 $NaHCO_3^-$ bubbled with 5% CO_2. For "0 Na^+" solutions, $NMDG^+$ replaced Na^+ and choline-HCO_3^- replaced $NaHCO_3^-$

Fig. 4. Addition of HCO_3^-/CO_2 at constant bath pH causes a hyperpolarization and a transient acidification of pH_i. The top and bottom traces were recorded simultaneously with a double-barreled pH-sensitive microelectrode. At a constant bath pH of 7.5, the superfusing solution was switched from a HEPES-buffered Ringer's solution (solution 1) to a solution buffered with 12.5 mmol/l HCO_3^-, 2.3% CO_2 (solution 3).

Fig. 5. Addition of HCO_3^-/CO_2 at constant bath pH increases the rate of recovery from acidification. The top and bottom traces were recorded simultaneously with a double-barreled pH-sensitive microelectrode. The initial Ringer's solution was solution 1 (pH 7.5). Cells were acidified by addition and withdrawal of 15 mmol/l NH_4^+, as indicated by the solid bar; NH_4^+ replaced Na^+. After acidification and partial recovery the buffer was changed at constant bath pH from 5 mmol/l HEPES (solution 1) to 12.5 HCO_3^-, 2.3% CO_2 (solution 3).

Fig. 6. In the presence of HCO_3^-/CO_2, removal of Na^+ reversibly blocks recovery from acidification and depolarizes the glial cell. The top and bottom records were recorded simultaneously with a double-barreled pH-sensitive microelectrode. The experiment was performed in HCO_3^-/CO_2-buffered Ringer's solution (solution 3, pH 7.5) and the cell was acidified by addition and withdrawal of 15 mmol/l NH_4^+ (NH_4^+ replaced Na^+). During the recovery from acidification, Na^+ was removed by replacing NaCl with NMDG-Cl and $NaHCO_3^-$ with choline-HCO_3^-

after the peak (Astion et al., 1989a) this procedure tends to underestimate the effect. During the recovery from acidification, 12.5 mmol/l HCO_3^-, 2.3% CO_2 was added at constant bath pH (7.5); after a transient acidification, HCO_3^-/CO_2 increased the initial linear rate of the pH_i recovery by a factor of 2.4. In four experiments, HCO_3^-/CO_2 increased the rate of pH_i recovery by an average factor of 2.3 (SD = 0.4, n = 4).

The dependence of the pH_i recovery on external Na^+ is shown in Fig. 6. Removal of Na^+ (replacement with $NMDG^+$ and choline) reversibly blocked the recovery from acidification, and was associated with a depolarization of the membrane. The depolarization preceded the decrease in pH_i and therefore was not due to a pH-sensitive conductance.

Fig. 7 shows a representative experiment in which SITS (0.5 mmol/l) was added to the HCO_3^-/CO_2-buffered Ringer's solution during the recovery from acidification. Addition of SITS first depolarized the membrane, and then blocked the recovery of pH_i.

If electrogenic Na^+/HCO_3^- cotransport contributes to the pH_i recovery after acidification, then the recovery should be accompanied by a hyperpolarization as Na^+, HCO_3^- (or its equivalent), and net negative charge enter the cell. We did not observe a hyperpolarization during recovery from acidification in the normal HCO_3^-/CO_2-buffered Ringer's solution (solution 3). However, it is quite possible that the inward movement of negative charge was present but unmeasurable. This is because, in the normal Ringer's solution, the glial K^+ conductance is so large that it can mask the relatively small voltage change resulting from the cotransporter current. To circumvent this problem, we decreased the K^+ conductance by performing experiments in the presence of 2 mmol/l Ba^{++} (Astion et al., 1989b). The results of a typical experiment are shown in Fig. 8. The glial cell was exposed to 15 mmol/l NH_4^+ on three occasions, twice in the absence and once in the presence of 12.5 mmol/l HCO_3^-, 2.3% CO_2. In HCO_3^-/CO_2, the withdrawal of NH_4^+, which will acidify the cells, produced an initial hyperpolarization of the membrane that overshot the original (pre-NH_4^+) baseline by about 5 mV. In the absence of HCO_3^-/CO_2, the withdrawal of NH_4^+ was not associated with a rapid, overshooting, hyperpolarization.

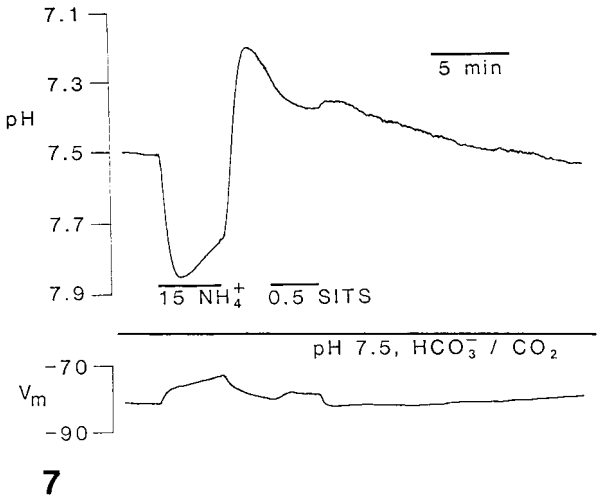

Fig. 7. Effect of SITS (0.5 mmol/l) on recovery from acidification in HCO_3^-/CO_2-buffered solution. The top and bottom records were recorded simultaneously with a double-barreled pH-sensitive microelectrode. The experiment was performed in HCO_3^-/CO_2-buffered Ringer's solution (solution 3, pH 7.5) and the cell was acidified by addition and withdrawal of 15 mmol/l NH_4^+ (NH_4^+ replaced Na^+). Addition of SITS (4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid) (0.5 mmol/l) led to a depolarization followed by a reversible inhibition of the recovery from acidification.

DISCUSSION

Results of Conventional Microelectrode Studies

The hypothesis of electrogenic Na^+/HCO_3^- cotransport (stoichiometry of HCO_3^-:Na^+ greater than 1) predicts a HCO_3^--induced hyperpolarization that is Na^+-dependent, SITS-sensitive, Cl^--independent, and not due solely to Na^+/K^+ ATPase activity or pH-sensitive conductances. The recordings of membrane potential from glial cells of *Necturus* optic nerve are consistent with these criteria. Previous studies have shown that the hyperpolarization induced by addition of HCO_3^- is Na^+-dependent, SITS-sensitive (Astion et al., 1989b), and insensitive to the removal of Cl^- (Astion et al., 1987). The hyperpolarization is not due to activation of the electrogenic Na^+/K^+ ATPase (Fig. 2). There is strong evidence that pH-sensitive conductances, which might be altered by CO_2 movements, do not contribute significantly to the HCO_3^--induced hyperpolarization because this would require that the pH-sensitive conductances be Na^+-dependent and SITS-sensitive, and give hyperpolarizations in response to both intracellular acidification (HCO_3^-/CO_2 added at constant pH; Figs. 2–4) and intracellular alkalinization (HCO_3^- added at constant CO_2; Fig. 1). A change in Ba^{++} binding is unlikely to contribute to the hyperpolarization for similar reasons. The lack of significant Ba^{++} involvement is also strongly suggested by the Na^+-dependent, HCO_3^--induced hyperpolarization that occurs in the absence of Ba^{++} (Fig. 3).

Further Evidence for Electrogenic Na^+/HCO_3^- Cotransport: Studies With pH-Sensitive Microelectrodes

In Figs. 5–8, we studied the ionic dependence and pharmacology of the recovery from acidification. All the results are consistent with the hypothesis that the electrogenic Na^+/HCO_3^- cotransporter functions in acid extrusion. The following is a summary of our major findings: 1) addition of HCO_3^- stimulates recovery from acidification and hyperpolarizes the cell (Fig. 5); 2) in the presence of HCO_3^-/CO_2, recovery from acidification is blocked by Na^+ removal; the removal of Na^+ also induces a rapid depolarization (Fig. 6); 3) in the pres-

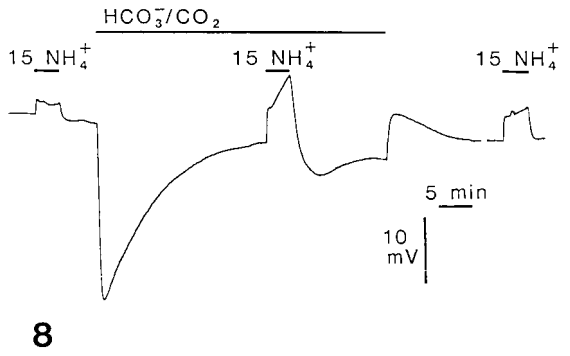

Fig. 8. NH_4^+-induced membrane potential changes in the presence and absence of HCO_3^-/CO_2; experiment done in the presence of 2 mmol/l Ba^{++}. NH_4^+ (15 mmol/l) was added (NH_4^+ replaced Na^+) for approximately 4 min in either the presence or absence of HCO_3^-/CO_2. The nominally HCO_3^--free solution was solution 2 (pH 7.5) and the HCO_3^-/CO_2-buffered solution was solution 4 (pH 7.5, 12.5 mmol/l HCO_3^-, 2.3% CO_2). As shown in the previous three figures, withdrawal of NH_4^+ acidifies the cells. In the presence of HCO_3^-/CO_2, the withdrawal of NH_4^+ is associated with a rapid, overshooting, hyperpolarization that slowly recovers toward the original baseline.

ence of HCO_3^-/CO_2, recovery from acidification is SITS-sensitive; addition of SITS during the recovery depolarizes the cell (Fig. 7); and 4) recovery from acidification is associated with a HCO_3^--dependent inward flux of negative charge (Fig. 8).

The observation that Na^+ removal blocks pH recovery (Fig. 6) raises the question as to the relative contribution of the Na^+/H^+ exchanger (Astion et al., 1989a) and the Na^+-HCO_3^- cotransporter to this process. It can be seen in Fig. 5 that the addition of HCO_3^-/CO_2 speeds up recovery in the presence of the Na^+/H^+ exchanger and moreover that the addition of SITS (Fig. 7), which blocks the cotransporter and not the exchanger, decreases the rate of pH_i recovery following an acid load. We conclude that both processes contribute to recovery, but these experiments do not permit us to quantitate their relative contributions.

An alternative hypothesis that can explain the above results is a Na^+-dependent Cl^-/HCO_3^- exchanger (for review see Boron, 1986) in combination with a Cl^- conductance. We consider the evidence for the two processes separately. Na^+-dependent Cl^-/HCO_3^- exchange is a well-known acid extrusion mechanism. It is an electroneutral process that moves Na^+ and more than one HCO_3^- (or its equivalent) in one direction and Cl^- in the opposite direction. The recovery from acidification observed in Figs. 5–7 was HCO_3^- and Na^+-dependent, and SITS-sensitive. These results are completely consistent with the existence of the Na^+-dependent Cl^-/HCO_3^- exchanger in the glial membrane. However, since the process is electroneutral, it is unable, by itself, to account for the membrane potential changes observed in the present study.

The Na^+-dependent Cl^-/HCO_3^- exchanger could induce the changes in membrane potential if there also existed a sufficiently large Cl^- conductance. This is not the case. In the absence of Ba^{++}, rapid removal of extracellular Cl^- leads to negligible changes in membrane potential (Astion and Orkand, unpublished observations). Thus, it is unlikely that the quantitative contribution of Cl^- to the membrane potential is sufficient to produce the membrane potential changes observed in the studies of glial pH_i. We therefore conclude that, in glial cells of *Necturus* optic nerve, electrogenic Na^+/HCO_3^- cotransport contributes to the recovery from a 0.2–0.3 pH unit acidification from the steady-state.

Glial vs. Renal Electrogenic Na^+/HCO_3^- Cotransport

Electrogenic Na^+/HCO_3^- cotransport was first described by Boron and Boulpaep (1983b) in renal proximal tubular cells of the tiger salamander. Since 1983, the process has been described in rabbit proximal tubule (Biagi and Sohtell, 1986; Grassl and Aronson, 1986; Sasaki et al., 1987; Soleimani et al., 1987), rat proximal tubule (Alpern, 1985; Yoshitomi et al., 1985), *Necturus* proximal tubule (Lopes et al., 1987), bovine corneal endothelium (Wiederholt et al., 1985), oxyntic cells of frog gastric fundus (Curci et al., 1987), and glial cells of the leech (Deitmer and Schlue, 1989).

The electrogenic Na^+/HCO_3^- cotransporter has been studied most thoroughly in the renal proximal tubule. In renal proximal tubule cells, the electrogenic Na^+/HCO_3^- cotransporter is localized in the basolateral membrane where it helps mediate the basolateral efflux (i.e., reabsorption) of HCO_3^-. In rat and rabbit proximal tubule cells, a stoichiometry of more than 2 HCO_3^- per Na^+ is required to allow the membrane potential to drive the efflux of Na^+ and HCO_3^- against their inwardly directed concentration gradients (Sasaki et al., 1987; Soleimani et al., 1987; Yoshitomi and Fromter, 1985; Yoshitomi et al., 1985). The most accurate study in rabbit suggests a 3:1 stoichiometry (Soleimani et al., 1987). Similarly, the stoichiometry is probably 3:1 in amphibian proximal tubule (Lopes et al., 1987). A precise determination of the stoichiometry in the current study requires knowledge of $[Na^+]_i$ and such data are not available for these glial cells.

The data in glial cells of *Necturus* optic nerve strongly suggest that the electrogenic Na^+/HCO_3^- cotransporter is poised to drive the pH_i to near the steady-state pH_i of approximately 7.4. Thus, the cotransporter responds to an acidification by mediating the influx of Na^+, HCO_3^-, and net negative charge. A similar behavior has been described for the electrogenic Na^+/HCO_3^- cotransporter of leech glia (Deitmer and Schlue, 1987, 1989). In contrast, the electrogenic Na^+/HCO_3^- cotransporter of the basolateral membrane of the renal proximal tubule is poised in quite a different manner; it tends to drive the pH_i to a level that is significantly acid to the steady-state pH_i of approximately 7.4 (Boron and Boulpaep, 1983a,b).

pH_i Regulation in Neuroglia

Glial cells of *Necturus* optic nerve have a pH_i of about 7.3 in the absence of HCO_3^-/CO_2 and about 7.4 in the presence of HCO_3^-/CO_2. To maintain this pH_i, acid must be actively regulated. Acid extrusion is probably accomplished by at least two mechanisms, Na^+/H^+ exchange (Astion et al., 1989a) and electrogenic Na^+/HCO_3^- cotransport. The data are also consistent with a Na^+-dependent Cl^-/HCO_3^- exchanger, but we have not been able to clarify the presence of this process.

Glial pH_i regulation has been studied most thoroughly in leech (Deitmer and Schlue, 1987, 1989) and cultured mouse oligodendrocytes (Kettenmann and Schlue, 1988). In addition, there have been preliminary reports on pH_i regulation in cultured rat astrocytes (Boyarsky et al., 1988; Dixon and Wilson, 1988). Like *Necturus*, leech glia extrude acid by means of a Na^+/H^+ exchanger and an electrogenic Na^+/HCO_3^- cotransporter (Deitmer and Schlue, 1987, 1989). The cotransporter of leech differs from *Necturus* in that the former is SITS-insensitive. Leech glia also have a third acid extrusion mechanism that is Na^+ and HCO_3^--dependent and SITS-sensitive, perhaps a Na^+-dependent Cl^-/HCO_3^- exchanger. In cultured mouse oligodendrocytes acid efflux is mediated by a Na^+/H^+ exchanger and a Na^+ and HCO_3^--dependent mechanism that is stilbene-insensitive and Cl^--independent (Kettenmann and Schlue, 1988). The stoichiometry of the latter mechanism is not clear; no obvious electrogenicity was observed. In rat astrocytes, acid extrusion processes include a Na^+/H^+ exchanger (Dixon and Wilson, 1988) as well as a Na^+-dependent Cl^-/HCO_3^- exchanger (Boyarsky et al., 1988, and personal communication).

In summary, the results reported here, based on recordings with conventional microelectrodes and double-barreled pH-sensitive microelectrodes, provide additional evidence for the existence of electrogenic Na^+/HCO_3^- cotransport in glial cells of *Necturus* optic nerve. The data strongly suggest that the cotransporter helps maintain a pH_i that is about 1.3 units alkaline to the pH_i expected from a passive proton distribution.

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