Developmental changes in intracellular pH regulation in medullary neurons of the rat

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Nottingham, S., J. C. Leiter, P. Wages, S. Buhay, and J. S. Erlichman. Developmental changes in intracellular pH regulation in medullary neurons of the rat. Am J Physiol Regulatory Integrative Comp Physiol 281: R1940–R1951, 2001.—We examined intracellular pH (pHi) regulation in the retrotrapezoid nucleus (RTN), a CO2-sensitive site, and the hypoglossal nucleus, a nonchemosensitive site, during development (postnatal days 2–18) in rats. Respiratory acidosis [10% CO2, extracellular pH (pH o) 7.18] caused acidification opment (postnatal days 2–18) in the hypoglossal nucleus, a nonchemosensitive site, during development (postnatal days 2–18) in rats. Respiratory acidosis [10% CO2, extracellular pH (pH o) 7.18] caused acidification in the RTN at all ages. Recovery of pHi from acidification in the RTN, but hypoglossal nucleus and RTN neurons could regulate pHi during intracellular acidification at constant pHo in young animals, but as animal age increased, the slope of pHi recovery diminished. In animals older than postnatal day 11, the pHi responses to hypercapnia were identical in the hypoglossal nucleus and the RTN, but hypoglossal nucleus and RTN neurons could regulate pHi during intracellular acidification at constant pHo, at all ages. Recovery of pHi from acidification in the RTN was inhibited by amiloride, but was unaffected by DIDS, suggesting a role for Na+/H+ exchange. Hence, pHi regulation during acidosis is more effective in the hypoglossal nucleus in younger animals, possibly as a requirement of development, but in older juvenile animals (older than postnatal day 11), pHi regulation is relatively poor in chemosensitive (RTN) and nonchemosensitive nuclei (hypoglossal nucleus).

As in many biological systems, the neural circuitry responsible for respiratory control is subject to developmental changes. For example, the greatest increases in dendritic length, dendritic branching, and synapse formation occur during the first 3 wk of life in the nucleus tractus solitarii (NTS) and the ventrolateral medulla (VLM), two sites implicated in CO2 chemoreception (41). There are also developmental changes in the ventilatory response to CO2 during the postnatal period in rats. A ventilatory response to CO2 is present at birth, but during the first wk of life, the ventilatory effect of hypercapnia wanes and reaches a nadir at ∼8 days of age. After this period, the hypercapnic ventilatory response increases and stabilizes at ∼2–3 wk of age (3, 48). Moreover, the pattern of breathing in whole animals during hypercapnia in the neonate differs from that in the adult rat (27). Neonatal animals predominantly increase tidal volume during hypercapnia, rather than breathing frequency, but adult rats increase breathing frequency and tidal volume (24). In addition to the changes in whole animal ventilatory control, there may be developmental changes in chemosensory function of individual neurons. Wang and Richerson (55) found marked developmental changes in spontaneous neuronal firing of cells located in one chemosensitive site, the medullary raphé. The firing rate of only 3% of the cells in the raphé increased in response to CO2 in rats at postnatal day 9, but in 18% of the raphé cells in rats at postnatal day 14, the firing rate increased during hypercapnia. These findings from in vivo and in vitro studies of respiratory control suggest that the neural circuits and the ionic currents that underlie CO2 chemosensory responses undergo significant postnatal development.

In most vertebrate cells, pHi regulation after acidification is achieved by membrane-bound transport systems, often including an amiloride-sensitive Na+/H+ exchanger (NHE) and one or more HCO3−-dependent transporters (5, 38). In contrast, pHi regulation after acid loading in medullary neurons prepared from neonatal rats (postnatal days 0–12) is simplified and involves only NHE (45). Furthermore, pHi regulation...
mediated by NHE in medullary neurons in two chemosensitive sites, the NTS and the VLM, was fully inhibited when extracellular pH (pHe) was <7.0, but proton transport in neurons located in two nonchemosensitive sites, the hypoglossal nucleus and inferior olive, was fully inhibited only when pHe was <6.7. These latter two sites are nonchemosensitive, in that CO2 or acid stimulation of these nuclei does not influence respiratory rhythm generation. Thus two processes contribute to the loss of ability to regulate pH in response to acidification in chemosensitive cells: chemosensitive neurons have only a single transporter (NHE) capable of responding to acidification, and the activity of this transporter is exquisitely sensitive to inhibition by reduced pHe, such as seen during even mild hypercapnic acidosis. It has been suggested that the foregoing pH regulatory characteristics may enable cells to operate as CO2 chemoreceptors (8, 13, 44, 45).

The dichotomous pattern of pH regulation among brain stem nuclei in brain slices from neonatal rats may be at odds with the pattern of pH regulation in vivo in the brain of adult animals, in which a more homogeneous pattern of poor pH regulation occurs. 31P nuclear magnetic resonance (NMR) spectroscopy measurements of pH in the brain of adult rats during sustained hypercapnia demonstrated surprisingly poor pH regulation (33). The rats were anesthetized, and the spectroscopic measurements provided an average pH value, derived from neurons and glia, for the entire brain beneath the NMR probe, which was over the midline of the posterior skull. Hence, regional variation of pH regulation may be masked by these NMR measurements, but pH regulation was, nonetheless, absent when animals inspired 5% CO2 and slow and incomplete when animals inspired 10% CO2 for a sustained period (3 h) even in nonchemosensitive regions of the brain.

We reevaluated pH regulation during hypercapnia in brain stem slices prepared from rats over a larger range of ages (postnatal days 2–18) to try to reconcile the divergent patterns of pH regulation in neonatal and adult animals and to test the hypothesis that the pattern of pH regulation evolved over the course of development. We examined pH regulation during hypercapnia in one chemosensitive nucleus and one nonchemosensitive nucleus within the brain stem. It was our hypothesis that pH regulation would remain consistently poor in the chemosensitive brain stem nucleus but change in the nonchemosensitive nucleus from a pattern of effective pH regulation early in development to a pattern of poor pH regulation in older animals.

METHODS

Slice Preparation

Medullary tissue slices were prepared from 2- to 18-day-old Sprague-Dawley rat pups of either gender, as previously described (52). These procedures were performed in accordance with the guidelines stated in the Guide for the Care and Use of Laboratory Animals as put forth by the Public Health Service, National Institutes of Health. Briefly, the animal was killed by rapid decapitation, and the brain was quickly removed from the skull and submerged in chilled (6–8°C) control saline (see below) for 2–3 min. The caudal cerebellum was removed, and a tissue block was prepared from the medulla oblongata. Transverse medullary slices (120- to 150-μm thick) from a region extending rostrally from the obex to the pons were sectioned in chilled, oxygenated control saline using a Vibratome and transferred to a holding chamber containing control saline at room temperature (22°C).

Solutions

For the medullary brain slice, the control, CO2/HCO3
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saline contained (in mM) 124 NaCl, 5 KCl, 2.4 CaCl2, 1.3 MgSO4, 1.24 KH2PO4, 26 NaHCO3, and 10 glucose. Control saline was equilibrated with 95% O2-5% CO2 to maintain a pH of 7.48 at 37°C. Solutions simulating respiratory acidosis were prepared by varying the concentration of CO2 while maintaining the HCO3
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concentration of the saline solution constant at 26 mM. Hypercapnic solutions were equilibrated with 10% CO2-90% O2. The pH of the hypercapnic saline solution was 7.18 at 37°C. The osmolarity for all solutions was ~300 mosm. NH4Cl solutions for prepulse experiments were prepared by replacing 75 mM NaCl isosmotically with NH4Cl. The HCO3
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concentration was not changed during the ammonia prepulse experiments. In the control solutions for the NH4Cl prepulse experiments, NaCl was replaced with 75 mM N-methyl-D-glucamine (NMDG) chloride. Amiloride was dissolved in dimethyl sulfoxide and used at a final concentration of 0.25–0.5 mM (the final dimethyl sulfoxide concentration in the perfusate was ~<0.1%). DIDS was dissolved in the solutions at a final concentration of 0.5 mM. In Na+-free, CO2-equilibrated solutions, NaCl was replaced with NMDG chloride, NaHCO3 was omitted, and the solution was gassed with 5% CO2 for 30 min and titrated to pH 7.48 with NMDG. Before delivery to the tissue, all solutions were loaded into 60-ml glass syringes. Solutions placed in these syringes were stable for up to 1 h; however, they were used immediately after they were loaded into the syringes.

The nigericin calibration solution contained (in mM) 104 KCl, 2.4 CaCl2, 1.3 MgSO4, 1.24 KH2PO4, 10 glucose, 25 NMDG-HEPES, 25 K-HEPES, and 4 × 10−3 nigericin. A calibration curve was constructed by titrating the pH of the calibration solutions from 5.8 to 8.6 pH units with KOH or HCl. A one-point calibration was performed at the end of each experiment using nigericin solutions that were titrated to pH 7.3 at 37°C (6). After each calibration, the entire perfusion apparatus was soaked and washed three times in 70% ethanol in water and subsequently rinsed repeatedly in distilled water to remove any retained nigericin. All reagents were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Superfusion Chamber

Single brain slices loaded with 2,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; see below) were removed from the holding chamber and transferred to a superfusion chamber described previously (46). Brain slices were immobilized in the chamber using a platinum wire and nylon grid (12). The total chamber volume was ~1 ml, and the chamber was continuously superfused with control or test solutions using syringe pumps at a rate of ~2 ml/min. The time required for complete exchange of the chamber solution was ~1 min. Solutions entering the chamber were heated to 37°C using a servo-controlled stage heater (WPI, Sarasota, FL),
and a continuous stream of 95% O2-5% CO2 was directed over the air-fluid interface. The pH of superfused solutions was monitored using a pH electrode (Beetwod, WPI).

Measurement of pHi

Slices were loaded with the pH-sensitive fluoroprobe BCECF (Molecular Probes, Eugene, OR) by incubation in a 20 μM solution of BCECF-AM in control saline bubbled with 95% O2-5% CO2 for 20–30 min at room temperature. BCECF-loaded slices were transferred to a holding chamber containing control saline (no dye) bubbled with 95% O2-5% CO2 at room temperature and maintained in this solution in the dark until the time of experimentation. The pH was measured in single, BCECF-loaded slices placed in a chamber mounted on the stage of an upright microscope (model E600FN, Nikon, Melville, NY) and continuously superfused with control saline. The pHi of individual neurons was measured within discrete areas (~0.042 mm2 at ×400 magnification) of the medulla by alternately exciting the area with light from a 75-W xenon lamp (Chiu Industries, Kings Park, NY) at wavelengths of 500 ± 10 and 440 ± 10 nm for 0.3–0.8 s. The excitation filters were switched by a computer-controlled filter wheel (Lambda 10-2, Sutter Instruments, Novato, CA). The emitted fluorescence from the intracellular BCECF was filtered using a 510-nm dichroic mirror (model C5700, Nikon), intensified, and measured with a cooled charge-coupled device camera (Photometrics, Tucson, AZ). The digital images were acquired and processed with Axon Imaging Workbench software (Axon Instruments, Foster City, CA). The rate of BCECF leakage is a measure of neuronal viability (4), and a rate of leakage <1%/min is consistent with viable neurons. The average rate for BCECF loss in our cells (measured as the rate of fall of the pH-insensitive fluorescence at 440-nm excitation) was ~0.3%/min, and results from neurons in which the rate of BCECF leakage exceeded 1%/min were not analyzed.

Data Analysis

Data were collected from ~160 brain slices from 80 animals. Steady-state pHi values were obtained by averaging at least five contiguous values at a point in the pHi vs. time record when pHi was stable. The initial fall in pHi after acidification was measured as the lowest value within 4 min of changing the perfusate from the control to the hypercapnic superfusate. We estimated the intrinsic buffer power (βint) of neurons by calculating the ratio of the change in intracellular bicarbonate to the change in pHi after changing from the control superfusate (pHi, 7.48) to the hypercapnic superfusate (pHi, 7.18). Intracellular bicarbonate was estimated using the Henderson-Hasselbalch equation, a pKb for carbonic acid of 6.12, a CO2 solubility of 0.03 mM/mmHg, and the measured pHi. Bicarbonate buffering was calculated by multiplying the estimated intracellular bicarbonate concentration by 2.303. Bicarbonate buffering power at the midpoint of pHi recovery during hypercapnic acidosis was calculated using the Henderson-Hasselbalch relationship and the pHi at the midpoint of pHi recovery: log[(10initial pHi + 10final pHi)/2], where initial and final refer to the end points of the linear portion of pHi recovery. The total buffer power was the sum of βint and the bicarbonate buffering power. The proton flux at the midpoint of the hypercapnic exposure (JH,mid) was calculated by multiplying the total buffering power at the midpoint of recovery by the rate of pHi recovery (38, 47). Recovery rates of pHi were calculated by performing least-squares regression on the linear portion of the pHi vs. time traces (Fig. 1). Values are means ± SE. Statistical comparisons were made using a repeated-measures or factorial ANOVA, as appropriate, in the General Linear Model procedure of Systat (SPSS Science, Chicago, IL). P ≤ 0.05 was considered statistically significant.

RESULTS

pHi, βint, and pHi Recovery in the Hypoglossal Nucleus and RTN

The time course of pHi changes during a typical hypercapnic experiment is shown in Fig. 1. The patterns of pHi changes in five representative neurons from the hypoglossal nucleus at postnatal day 4 and five neurons from the RTN at postnatal day 7 are shown during the control period and during hypercap-
Developmental changes in medullary pH regulation.

All the neurons in the hypoglossal nucleus show pHi recovery during hypercapnic acidosis, but the majority of neurons in the RTN show no pHi recovery. The average steady-state control pHi values measured in neurons from the hypoglossal nucleus at all ages (postnatal days 2–16) and from the RTN at all ages (postnatal days 4–18) are shown in Table 1. All measurements were made at pHo 7.48 at 37°C in 26 mM HCO3−-buffered solution equilibrated with 5% CO2. The average steady-state pHi in the hypoglossal nucleus was 7.18 ± 0.01 (n = 73 cells), and there was no significant effect of developmental age on pHi (P = 0.86). The average steady-state control pHi in the RTN was 7.19 ± 0.02 (n = 88 cells). There was no effect of developmental age on pHi (P = 0.45). The control pHi values were not different in the hypoglossal nucleus and the RTN (P = 0.72).

The initial effects of exposing cells in each nucleus to hypercapnia (10% CO2, pHo 7.18) are summarized in Table 2. Exposing the hypoglossal nucleus to solutions mimicking hypercapnic acidosis (10% CO2, pHo 7.18) decreased pHi to 7.03 ± 0.01. The extent of acidification during hypercapnia was not dependent on age (P = 0.18). Exposing RTN neurons to solutions mimicking hypercapnic acidosis decreased pHi to 6.98 ± 0.01, but the extent of acidification during hypercapnia was dependent on age (P < 0.03). The pHi fell more in younger animals (younger than postnatal day 11; pHi 6.94 ± 0.01, n = 27 cells) than in older animals (postnatal day 11 and older; pHi 7.00 ± 0.02, n = 61). Although the control pHi values did not differ between the hypoglossal nucleus and the RTN, the fall in pHi during acidosis was significantly greater in the RTN than in the hypoglossal nucleus (P < 0.01).

Estimates of βint are summarized in Table 3. The estimates of βint were somewhat variable, as shown by the relatively large SE. However, significant differences existed between nuclei. The βint in the hypoglossal nucleus was 40.6 ± 1.7 meq·l−1·pH unit−1, and this value increased significantly as developmental age increased (P = 0.0001). The RTN had a lower βint (17.0 ± 1.1 meq·l−1·pH unit−1), but βint also increased in older animals (P = 0.0001). The rate at which βint increased as animals aged was similar in the hypoglossal nucleus and the RTN (there was no interaction between age and nucleus, P = 0.53), even though the absolute value of βint at any age was lower in the RTN than in the hypoglossal nucleus (P < 0.0001).

There were major age-related differences in the rate of pHi recovery and the rate of proton flux between nuclei and among animals of different ages. The rates of pHi change during sustained hypercapnia are shown for the hypoglossal nucleus and RTN in Fig. 2. Recovery of pHi was apparent in the hypoglossal nucleus in younger animals, but the rate of recovery diminished as animal age increased. The loss of pHi recovery as a function of age was highly significant (P < 0.0001). In contrast, there was little or no evidence of pHi recovery in the RTN at any age studied and no age-related change in the slope of pHi recovery (P = 0.38).

Because βint differed between nuclei and changed as a function of animal age, the absolute values of the slope of pHi change during hypercapnic acidosis might not accurately depict proton flux rates (JH,mid), which depend on the total buffering power (βint + bicarbonate buffering) and the rate of change of pHi. Therefore, we estimated JH,mid during hypercapnic acidosis in the hypoglossal nucleus and RTN at different postnatal ages.
Effect of pHo on pHi Regulation

In previous studies of pH recovery during acidosis in CO2-chemosensitive and nonchemosensitive regions in the brain stem in younger animals (postnatal day 12 and younger), pH regulation was inhibited when pHo was also reduced, but pHi regulation was apparent when only pHi (and not pHo) was reduced by the acidic stress (45). We examined the effect of pHo on pHi regulation during hypercapnic acidosis (pHi and pHo drop) and after an ammonia prepulse (pHi falls, but pHo remains constant) in the hypoglossal nucleus and RTN of animals at postnatal days 16 and 17, respectively. The pH profile over the course of the experiment and the sequence of test conditions are shown in representative neurons from both nuclei in Fig. 3. We used $\beta_{int}$ calculated from the initial change in pHi and calculated the change in bicarbonate during the hypercapnic acidosis to derive an estimate of $\beta_{int}$, and we measured the rate of pHi change per minute during the linear portion of pHi “recovery” during hypercapnia and after the ammonia prepulse. From these measurements, we calculated $J_{H,mid}$ during each period of acidosis in each of the nuclei. The average proton flux rates were $0.001 \pm 0.038$ meq·l$^{-1}$·min$^{-1}$ during hypercapnia in the hypoglossal nucleus ($n = 12$) and $-0.033 \pm 0.032$ meq·l$^{-1}$·min$^{-1}$ in the RTN ($n = 16$). These values are not significantly different from each other ($P > 0.5$). Compared with the hypercapnic $J_{H,mid}$, the average $J_{H,mid}$ after the prepulse increased to $1.316 \pm 0.072$ meq·l$^{-1}$·min$^{-1}$ in the hypoglossal nucleus and $0.800 \pm 0.063$ meq·l$^{-1}$·min$^{-1}$ in the RTN. Both values significantly exceed the $J_{H,mid}$ during hypercapnia ($P < 0.001$ in both cases). Furthermore, the $J_{H,mid}$ was significantly greater in the hypoglossal nucleus than in the RTN after the ammonia prepulse ($P < 0.001$), even though the rates were similar during hypercapnia. The pHi was significantly lower at the midpoint of the linear portion of the pHi recovery phase in the RTN than in the hypoglossal nucleus ($P < 0.001$), which is consistent with the lower buffering power in the RTN shown in Tables 1–3. However, within each nucleus, pHi was similar during hypercapnia and after the NH4Cl prepulse protocol. Therefore, it appears that the inhibitory effect of pHo on pHi regulation was greater in the hypoglossal nucleus, since $J_{H,mid}$ increased more than in the RTN when pHi was held constant after the ammonia prepulse and the inhibitory effect of pHo was absent.

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Regulation of pHi in the RTN

The mechanisms of pHi regulation have been studied in the hypoglossal nucleus and inferior olive (nonchemosensitive regions) and in two medullary regions containing putative CO2 chemosensory neurons: the rostral VLM and the NTS (44, 45). In all four regions, an NHE mechanism dominated pHi recovery during acidosis. The NHE regulation in these CO2 chemosensory sites demonstrated unusual sensitivity to pHo, which inhibited the activity of the NHE mechanism. The pHi regulatory mechanisms in the RTN, also a putative site of CO2 chemosensory neurons, have not been described. Therefore, we tested the hypothesis that NHE also regulates pHi in the RTN under control conditions and during intracellular acidosis to determine whether the association of NHE inhibition and CO2 chemosensitivity is a more general phenomenon.

We defined the RTN as a thin layer of cells in the VLM extending from the rostral tip of the facial nucleus caudally to the rostral retrofacial nucleus and lying within 400–600 μm of the ventral medullary surface in its most rostral extent and within 100–300 μm of the ventral medullary surface at its most caudal extent. The RTN is bounded laterally by the spinotrigeminal tract and medially by the parapyramidal region (30).

Effects of amiloride on pHi recovery in RTN neurons.

We examined the effect of amiloride on pHi regulation during normocapnic control conditions (5% CO2, pHo 7.48) and hypercapnic conditions (10% CO2, pHo 7.18). The responses of pHi to these conditions in representative neurons are shown in Fig. 4 (normocapnia) and Fig. 5 (hypercapnia). In both cases, amiloride significantly reduced the rate of pHi recovery. We estimated the rate of pHi change during the linear portion of each test condition: normocapnia during and after amiloride exposure and hypercapnia before and after amiloride exposure. We do not have an estimate of βint for the neurons tested under normocapnic conditions, but for this analysis, we assumed that βint was similar in the normocapnic and hypercapnic neurons. We compared JH,mid during normocapnia after amiloride was removed from the perfusate with JH,mid during hypercapnia before amiloride was added to the perfusate. The proton flux rate was significantly greater during normocapnia (1.09 ± 0.09 meq·l−1·min−1, n = 11) than during hypercapnia (−0.29 ± 0.09 meq·l−1·min−1, n = 12, P < 0.001). The pHi was actually lower during normocapnic pHi recovery after treatment with amiloride (Fig. 4) than during the initial phase of hypercapnia (Fig. 5). This results in a lower intracellular bicarbonate concentration, a lower total buffering power, and a lower estimate of JH,mid during normocapnic recovery. However, JH,mid in the absence of amiloride...
was still greater during normocapnia than during hypercapnia, a finding that emphasizes the potent inhibitory effect of pHo on pHi regulation. The proton flux rate fell under both conditions when amiloride was added to the perfusate: $J_{H,mid} = -1.16 \pm 0.09 \text{ meq}^{-1} \text{min}^{-1}$ during normocapnia and $-1.06 \pm 0.08 \text{ meq}^{-1} \text{min}^{-1}$ during hypercapnia. These flux rates were significantly different from the flux rates without amiloride ($P < 0.001$) and significantly different from each other ($P < 0.001$). It is worth noting that an amiloride-sensitive mechanism was active during normocapnia, since amiloride caused pHi to fall. This is not the case in the rostral VLM or NTS, where amiloride caused no change in pHi under control normocapnic conditions (44).

We also examined the effect of amiloride on pHi regulation after an ammonia prepulse, which acidifies the neuron, but at a constant pHo. The pHi profile of a representative neuron in the RTN of an animal at postnatal day 18 is shown in Fig. 6. Comparisons were made within cells, and we set $\beta_{int}$ equal to the average value of neurons in the RTN in animals older than postnatal day 11 to calculate $J_{H,mid}$. The initial $J_{H,mid}$ was $0.026 \pm 0.002 \text{ meq}^{-1} \text{min}^{-1}$, and $J_{H,mid}$ fell significantly to $-0.001 \pm 0.001 \text{ meq}^{-1} \text{min}^{-1}$ when amiloride was present in the perfusate ($P < 0.001$, $n = 6$). The rate rose to $0.007 \pm 0.003 \text{ meq}^{-1} \text{min}^{-1}$ once amiloride was no longer present; this rate was significantly greater than the proton flux rate in the presence of amiloride ($P < 0.001$) but also less than the rate before application of amiloride when pHi was lower as well ($P < 0.001$).

**Effect of Na⁺-free solutions on pHi recovery in RTN neurons.** We examined the effect of removing extracellular Na⁺ on pHi regulation after an ammonia prepulse. The pHi profiles from representative neurons in the RTN from an animal at postnatal day 15 are shown in Fig. 7. We calculated $J_{H,mid}$ during the recovery from acidification after Na⁺ was removed and after Na⁺ was restored using $\beta_{int}$ derived from Table 3. During the initial pHi recovery phase before Na⁺ was removed, some of the neurons had not established a stable recovery rate, and data were not analyzed from this period of the study. The rate of proton flux when Na⁺ was removed was $-0.419 \pm 0.028 \text{ meq}^{-1} \text{min}^{-1}$ ($n = 14$). After Na⁺ was restored, the rate of change was $0.418 \pm 0.029 \text{ meq}^{-1} \text{min}^{-1}$. These rates of change...
are significantly different \((P < 0.001)\). Thus \(pH_i\) recovery during acidosis requires extracellular \(Na^+\). NHE, which requires extracellular \(Na^+\), is the primary exchange mechanism active during acidosis in many brain stem nuclei (44), and when NHE is present, but extracellular \(Na^+\) is removed, the exchanger may run in "reverse," effectively acid loading the cell.

**Effects of DIDS on \(pH_i\) regulation in RTN neurons.** In addition to NHE, many cells rely on a variety of bicarbonate transporters to regulate \(pH_i\). We examined the role of \(Cl^-/HCO_3^-\) exchange during acidic challenge by adding DIDS, a \(Cl^-/HCO_3^-\) exchange inhibitor, to the perfusate after an ammonia prepulse. The \(pH_i\) profile during this protocol is shown from a representative neuron from the RTN in an animal at postnatal day 13 in Fig. 8. The rate of proton flux was estimated from the rate of \(pH_i\) change and \(\beta_{int}\) from Table 3. The estimated proton flux rate during recovery from the prepulse-induced acidosis was \(0.462 \pm 0.065\) meq\(\cdot l^{-1}\cdot min^{-1}\) when DIDS was present and \(0.520 \pm 0.084\) meq\(\cdot l^{-1}\cdot min^{-1}\) after DIDS was removed from the perfusate. These values are not significantly different \((n = 25, P = 0.521)\); therefore, there is no evidence of a DIDS-sensitive mechanism of \(pH_i\) regulation active during recovery from acidosis in these neurons.

**DISCUSSION**

There are three main findings in this study. First, \(pH_i\) regulation was more effective in younger animals (no older than postnatal day 11) in the hypoglossal nucleus, as Ritucci et al. (44, 45) previously described. Second, as animals aged, \(pH_i\) regulation deteriorated in the hypoglossal nucleus, and after postnatal day 11, \(pH_i\) regulation was similar during hypercapnia in all medullary nuclei tested regardless of whether a nucleus was a putative chemosensory region. The reduced capacity for \(pH_i\) regulation in the hypoglossal nucleus was associated with the emergence of inhibition of \(pH_i\) regulation by \(pH_e\). Finally, \(pH_i\) regulation in the RTN seemed to depend on an NHE mechanism that is similar to the mechanisms described for other chemosensory areas of the brain stem (44, 45).

**Critique of Methods**

The interpretation of our results relies on estimates of proton flux. These estimates, in turn, depend on the directly measured rate of \(pH_i\) change per unit time and the calculated total buffer power \((\beta_{int} + \text{bicarbonate buffering})\). Calculations of \(\beta_{int}\) vary significantly in different tissues and organisms (47). The variability arises, in part, from the particular method of acid loading used to determine \(\beta_{int}\), and using CO2 as the acid stress, instead of an ammonia prepulse or propionate, leads to the lowest estimates of \(\beta_{int}\) among these possible acid-loading protocols (22). We did not modify the perfusate constituents or add inhibitors of \(pH_i\) regulation (i.e., amiloride or stilbene derivatives) when we made estimates of \(\beta_{int}\), and we did not project the linear portion of the \(pH_i\) profile during hypercapnia back to time \(0\) to try to estimate the change in \(pH_i\) associated with hypercapnia more accurately as some investigators have done (47). Nonetheless, our estimates of \(\beta_{int}\) are similar to other estimates of neuronal \(\beta_{int}\) when hypercapnia was used to estimate \(\beta_{int}\) (22, 47). This probably occurs because we measured the hypercapnic fall in \(pH_i\) within 4 min after hypercapnia was applied, and this is close to time \(0\), since some of the delay in the fall in \(pH_i\) after the stimulus was applied reflects the washin time of our perfusate chamber and the tissue slice. Furthermore, the fall in \(pH_i\) associated with hypercapnia can inhibit \(pH_i\) regulatory mechanisms and limit the effect of proton or bicarbonate transport mechanisms on \(pH_i\) in the short time between application of the stimulus and the measurement of the initial \(pH_i\) value used to calculate \(\beta_{int}\). Thus our calculated values of \(J_{H/+mid}\) are derived from estimates of buffering power, but they are likely to provide an accurate representation of proton fluxes in the conditions we studied.

Within the brain slice, there are glia and neurons. We are confident that cells in which we measured \(pH_i\) are neurons. On the basis of electrophysiological and immunohistochemical criteria, we showed previously that the BCECF-loaded cells in which we determine \(pH_i\) are neurons rather than glia (46). Furthermore, cells loaded with BCECF after the procedure we used are rapidly killed by the neurotoxin kainic acid (data not shown). Although glia do have glutamate receptors, exposure to kainic acid is not lethal to glia (17). Finally, we should note that the RTN is a heterogeneous nucleus with multiple neuronal types. Our conclusions refer only to the average responses of all neurons within the RTN; we have no information about differences that may exist between neurons within the RTN or the hypoglossal nucleus.
Steady-State pH\textsubscript{i} Measurements

There were no systematic differences in neuronal pH\textsubscript{i} between the putative CO\textsubscript{2}-sensitive nucleus (RTN) and the CO\textsubscript{2}-insensitive nucleus (hypoglossal nucleus). Ritucci et al. (45) obtained a similar result when studying the inferior olive and hypoglossal nucleus (CO\textsubscript{2} insensitive) and the NTS and rostral ventral medulla (CO\textsubscript{2} sensitive). We did find differences in $\beta_{\text{int}}$ between the RTN and the hypoglossal nucleus and between early and late developmental ages of both nuclei. Buckler et al. (8) found relatively low $\beta_{\text{int}}$ in isolated type I cells of the carotid body, which are also CO\textsubscript{2} sensitive. A low buffering power permits changes in pH\textsubscript{i} to be reflected by similar changes in pH\textsubscript{o}, although the ultimate steady-state pH\textsubscript{i} will depend on the effectiveness of pH\textsubscript{i}-regulating mechanisms. Buckler et al. (8) speculated that the steep pH\textsubscript{i}-pH\textsubscript{o} relationship in the carotid body was an important aspect of acid sensing by this organ. In contrast, Ritucci et al. (44) found no differences in $\beta_{\text{int}}$ among the chemosensory and nonchemosensory nuclei they studied, and buffer power was not different between CO\textsubscript{2} chemosensory and nonchemosensory regions in the pulmonate snail Helix aspersa (18). Thus it seems that buffer power may be lower in some CO\textsubscript{2}-sensitive neurons (e.g., the RTN), and although this may enhance the sensitivity to pH\textsubscript{o} changes in the absence of effective pH\textsubscript{i} regulation, it is not an essential element in the chemosensory process. Finally, $\beta_{\text{int}}$ increased within the RTN and hypoglossal nucleus as the animals aged. Haworth et al. (19) observed a similar trend in ventricular myocytes over the course of neonatal development.

Steady-State pH\textsubscript{i} Regulation in RTN Neurons

Depending on the type of vertebrate neurons studied, some authors have attributed pH\textsubscript{i} regulation during acidification to a single proton transport system, NHE (16, 26, 28, 36, 51), while other authors implicated an additional role for HCO\textsubscript{3}\textsuperscript{-}-dependent acid extrusion mechanisms (9, 40, 42, 49). Inhibition of NHE resulted in progressive cellular acidification during steady-state and acidic conditions, indicating that RTN neurons are continuously exposed to a net acid load that is normally offset by acid extrusion of the NHE. Moreover, we found that this mechanism was dependent on extracellular Na\textsuperscript{+} and inhibited by amiloride, indicating that it is probably an NHE. Although we do not know the source of this net acidification in neurons, it may be associated with the metabolic generation of lactate (54). Dependence on NHE seems to be a common feature of central chemosensory nuclei; the RTN, NTS, and VLM seem to regulate pH\textsubscript{i} during acidosis by NHE (45).

Recovery of pH\textsubscript{i} During Hypercapnic Acidosis

We have shown that pH\textsubscript{i} recovery in CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}-buffered solutions is entirely Na\textsuperscript{+} dependent and inhibited by amiloride and, furthermore, does not appear to be affected by DIDS. Thus our findings are consistent with the presence of a single proton transport mechanism, NHE, which is responsible for pH\textsubscript{i} regulation in RTN neurons and possibly all medullary cells during acidosis. A similar dependence of pH\textsubscript{i} regulation on NHE has been described in cultured hippocampal neurons, cultured cortical and sympathetic neurons, and medullary neurons (26, 36, 40, 45, 51). However, pH\textsubscript{i} recovery in hippocampal and cortical neurons could not be completely inhibited by the application of amiloride, suggesting that there may be differential expression of NHE isoforms among regions within the brain (25).

Over the course of development, pH\textsubscript{i} regulation in the hypoglossal nucleus became less effective during hypercapnia. We believe that this reflects the emergence in older animals of a pH\textsubscript{i} regulatory mechanism that is increasingly sensitive to inhibition by pH\textsubscript{o}. Low pH\textsubscript{c} inhibits NHE activity (2), and susceptibility to inhibition by pH\textsubscript{o} may differ among NHE isoforms. Developmentally mediated changes in the expression of NHE isoforms 1 and 3 have been described in the brain, although not specifically in the medulla (25). Thus it is possible that the progressive decrease in pH\textsubscript{i} regulation during hypercapnic acidosis in hypoglossal neurons as animals aged reflected a change in NHE isoform expression. However, NHE mechanisms are also susceptible to modulation by a variety of second messengers (35), and a developmental change in second-messenger expression affecting NHE function might also increase the sensitivity of pH\textsubscript{i} regulation by NHE to pH\textsubscript{o}.

A possible alternate explanation for the decline in pH\textsubscript{i} regulatory capacity as animals age is a generalized loss of pH\textsubscript{i} regulatory capacity. For example, the number of NHE proteins per unit cell volume might decline as animals mature. We believe that this is unlikely, because older animals still regulate pH\textsubscript{i} well during intracellular acidosis when pH\textsubscript{o} is kept at the control value (Fig. 3), and the loss of pH\textsubscript{o} regulatory activity, therefore, seems specifically related to inhibition of pH\textsubscript{i} regulation by pH\textsubscript{o}. We do not have measurements of maximum pH\textsubscript{i} regulatory activity during acidosis as a function of age to address this issue. Studies to examine this hypothesis in which we measure $J_{\text{H}_{\text{mid}}}$ during isohydric hypercapnia or NH\textsubscript{3}Cl prepulse studies over the course of development are underway but beyond the scope of this report.

pH\textsubscript{i} as a Unique Characteristic of Chemosensory Function

In theory, a neuron with a single pH\textsubscript{i} regulatory mechanism inhibited by small decreases in pH\textsubscript{o} would make an ideal chemosensor of hypercapnic acidosis. Such a neuron would not “regulate” the stimulus, and decreases in pH\textsubscript{i}, associated with elevated CO\textsubscript{2} would be rapidly transduced into a sustained decrease in pH\textsubscript{i} that would show no accommodation during acidification (13, 45). Previous studies using brain slices from neonatal rats showed that neurons located in putative chemoreceptor sites within the medulla of young ani-
mals (postnatal days 0–11) failed to regulate pHᵢ in response to hypercapnic acidosis, whereas neurons in nonchemoreceptor sites showed rapid regulation toward normocapnic values (45). Hence, the pHᵢ responses to hypercapnia in chemosensitive regions of the brain stem are in keeping with the ideal theoretical design of a chemosensor, and nonchemosensory regions seemed to regulate pHᵢ well in animals younger than postnatal day 11. However, the most important finding in this study is our observation that pHᵢ regulation in chemosensitive and nonchemosensitive sites is not different among nuclei in juvenile animals (older than postnatal day 11). The hypoglossal nucleus, RTN, medial vestibular nucleus, and facial nucleus failed to show pHᵢ recovery during physiological levels of hypercapnic acidosis in older animals. Neurons studied in animals older than postnatal day 11 had similar pHᵢ responses to hypercapnic challenge regardless of the brain stem site tested. Thus the profile of pHᵢ regulation during hypercapnic acidosis in older animals is not a unique characteristic reflecting chemosensory function, and putative chemoreceptors and nonchemoreceptors are equally sensitive to the inhibitory effects of extracellular acidification in older juvenile animals. Our findings are consistent with previous studies measuring brain pHᵢ using spectroscopic techniques in which goats exposed to sustained hypercapnia (5 and 10% inspired CO₂) for several hours demonstrated a prompt fall in pHᵢ within the brain but little or no recovery of pHᵢ (33). In all CO₂ chemosensory cells studied thus far (8, 18, 44), pHᵢ regulation is inhibited when pHₑ falls. However, the results from the hypoglossal nucleus, facial nucleus, and medial vestibular nucleus in older animals indicate that the converse is not true; nonchemosensory neurons do not necessarily regulate pHᵢ well when pHₑ falls during hypercapnia. Thus poor pHᵢ regulation is not uniquely associated with CO₂ chemosensory neurons. Goldstein et al. (18) reached a similar conclusion in studies of CO₂ chemosensory and nonchemosensory regions of the pulmonate snail H. aspersa.

**Perspectives**

The emergence of poor pHᵢ regulation in nonchemosensitive regions of the brain stem as the animal ages is paradoxical given the important role of pHᵢ in a plethora of cellular processes (39). The pHᵢ, however, may modulate events associated with growth and mitosis. For example, intracellular alkalinization and effective pHᵢ regulation may be required for growth and development (23) or at least “permit” cellular processes important for growth and development (38). Hence, we suspect that neurons in younger animals, unless they must contribute to the CO₂ chemosensory response of the whole animal, will require effective pHᵢ regulation as a necessary function to maintain and promote development. As the animal ages and development is completed, the strategy of pHᵢ regulation may switch from a pattern essential for growth and development to a pattern better suited to enhance survival of terminally differentiated neurons without growth potential.

We do not believe that sustained acidification during hypercapnia in older animals evolved to serve the needs of chemosensitivity. We believe that sustained intracellular acidification of neurons during hypercapnic acidosis has a neuroprotective effect, and the evolution of this pattern of pHᵢ regulation in chemosensory neurons was secondary to its neuroprotective effects. Extracellullar and intracellular acidification are natural consequences of ischemia and brain injury, and neurons exposed to extracellular acidification during ischemic stress in vivo or hypoxic, anoxic, or traumatic stress in vitro swell and may lyse (15, 31). Cell death has been attributed to elevated levels of intracellular Ca²⁺ and intracellular Na⁺. Intracellular Ca²⁺ and, to a lesser extent, Na⁺ rise after excitotoxic glutamate receptor activation. Na⁺ also rises by virtue of the action of NHE activity in response to the intracellular acidosis. As a result of the increase in intracellular Na⁺, the Na⁺/Ca²⁺ exchanger may also operate in reverse and further load the cell with Ca²⁺. The extent of cell death can be minimized during conditions of extracellular acidification by blocking Na⁺ entry with Na⁻ substitues (15, 50) or specific blockers of Na⁺ channels (1, 7) or inhibitors of NHE such as amiloride (1, 14). In vivo studies of ischemia have demonstrated the efficacy of administration of such drugs in minimizing neuronal damage (37). Moreover, reducing pHₑ inhibits pHᵢ regulation and is neuroprotective in tissue culture models of ischemia (53). Thus we believe that the inhibition of NHE during hypercapnic acidosis is a mechanism whereby all neurons minimize the likelihood of cellular lysis and death during ischemia and anoxia. Our data suggest that this neuroprotective effect is differentially expressed over the course of development in different brain stem nuclei. Nuclei containing putative chemoreceptors appear to exhibit the proton inhibition of NHE early in life, a response that is also sensible in terms of the animal’s need to have a ventilatory response to CO₂ after parturition. Nonchemosensory neurons may be resistant to the inhibitory effects of pHₑ because of the need for effective pHᵢ regulation and alkalinization during processes of growth and development. However, as the animal ages and growth is less important in neurons, the neuroprotective effect of inhibiting NHE emerges and predominates, and pHₑ inhibits pHᵢ regulation during extracellular acidosis in older animals. Thus, once the brain stem has matured and established appropriate synaptic targets, the pattern of pHᵢ regulation changes to serve a neuroprotective function during periods of ischemia.

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REFERENCES


