Intracellular pH regulation in neurons from chemosensitive and nonchemosensitive regions of *Helix aspersa*

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Intracellular pH regulation in neurons from chemosensitive and nonchemosensitive regions of *Helix aspersa*. Am J Physiol Regulatory Integrative Comp Physiol 279: R414–R423, 2000.—We used 2’,7’-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), a pH-sensitive fluorescent dye, to study intracellular pH (pHi) regulation in neurons in CO2 chemoreceptor and nonchemoreceptor regions in the pulmonate, terrestrial snail, *Helix aspersa*. We studied pHi during hypercapnic acidosis, after ammonia pre-pulse, and during isohydric hypercapnia. In all treatment conditions, pHe fell to similar levels in chemoreceptor and nonchemoreceptor regions. However, pHi recovery was consistently slower in chemoreceptor regions compared with nonchemoreceptor regions, and pHe recovery was slower in all regions when extracellular pH (pHe) was also reduced. We also studied the effect of amiloride and DIDS on pHi regulation during isohydric hypercapnia. An amiloride-sensitive mechanism was the dominant pHi regulatory process during acidosis. We conclude that pHe modulates and slows pHi regulation in chemoreceptor regions to a greater extent than in nonchemoreceptor regions by inhibiting an amiloride-sensitive Na+/H+ exchanger. Although the phylogenetic distance between vertebrates and invertebrates is large, similar results have been reported in CO2-sensitive regions within the rat brain stem.

respiratory control; acid-base balance; central carbon dioxide chemoreceptors; invertebrates; snails

Since Winterstein first proposed his “reaction theory” of respiratory control in 1910 (32, 33) in which he attributed the excitatory effects of CO2 on ventilation to changes in hydrogen ion concentration, investigators have debated both the validity of the theory and the locus of excitation. Two issues pertain to the locus of excitation: where are CO2 chemoreceptors within the central nervous system and where is the pH that the chemosensors detect [extracellular pH (pHe), intracellular pH (pHi), the pHe-pHi gradient, etc.? We have explored these issues in an air-breathing invertebrate, *Helix aspersa* (12). Snails are phylogenetically distant from mammals, and aerial respiration evolved independently in vertebrates and invertebrates. Nonetheless, pulmonate, terrestrial snails developed remarkably similar central neural mechanisms to monitor CO2 and regulate ventilation as a function of CO2 and pHe (9–11, 13, 16). For example, exposure of the whole snail to CO2 increased opening of the pneumostome, a muscular aperture that regulates access to the gas exchange surface of the mantle cavity. Furthermore, we identified a discrete, CO2-sensitive region along the margins of the visceral and right parietal ganglia in the central nervous system of the snail that mediated responses of the pneumostome to CO2. Focal hypercapnic stimulation of this CO2-sensitive region increased pneumostomal opening and mimicked the response that we observed in intact snails exposed to ambient hypercapnic gases (11). We also identified intrinsically CO2-sensitive neurons within the CO2-sensitive region (14).

Within identified CO2 chemoreceptor regions, the location of the “CO2 receptors” (intracellular vs. extracellular) has not been defined in either vertebrates or invertebrates (17, 19). In *H. aspersa*, pHi, as opposed to pHe, or the pHe-pHi gradient, seems to be the essential stimulus of CO2 chemoreceptors (10). The evidence is less clear-cut in mammals, but available data are consistent with the hypothesis that mammalian CO2 chemoreceptors also respond to pHi (19). If pHi mediates the ventilatory effects of CO2, pHi regulation may differ between chemoreceptor and nonchemoreceptor cells. In theory, chemoreceptor neurons, unlike other cells, should not exhibit pHi recovery on acidification with CO2 so that the CO2-induced pHi change, the respiratory stimulus, does not diminish over time; the chemoreceptor stimulus should persist as long as the acidosis persists. On the other hand, robust pHi regulatory mechanisms may exist in nonchemoreceptor neurons to restore pHi during acidic stress and preserve protein and cellular function. Ritucci et al. (24) recently tested this hypothesis when they investigated the effects of hypercapnia on pHi regulation of neurons in medullary brain slices from preweanling Sprague-Dawley rats. Regulation of pHi differed between neurons in chemosensitive areas, the nucleus of the solitary tract (NTS) and ventrolateral medulla (VLM), and

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nonchemosensitive areas, the inferior olive and hypocentral nucleus of the medulla. A subset of neurons in the chemosensitive areas was unable to regulate pHᵢ when pHₑ and pHᵢ fell during acidic stimulation; whereas pHᵢ in neurons in nonchemosensitive areas recovered toward the initial, control pHᵢ although the acidic stress persisted. However, pHᵢ recovered in all areas during intracellular acidosis if pHₑ was not acidified. Furthermore, pHᵢ recovery from acidic stress in medullary neurons, whether in chemosensitive or nonchemosensitive regions, was due solely to an Na⁺/H⁺ exchange mechanism. These results support the hypothesis that chemoreceptor cells have relatively poor pHᵢ regulation but also indicate that the pattern of pHᵢ regulation was highly dependent on pHₑ. These findings are similar to the pattern of pHᵢ regulation during hypercapnia in isolated glomus cells of the carotid body from neonatal rats, which are also CO₂ sensitive (5, 6).

In this study, we compared pHᵢ regulatory function between neurons in the CO₂ chemosensitive region and neurons in nonchemosensitive regions in the subesophageal ganglia of H. aspersa. We measured the pHᵢ of individual neurons using the pH-sensitive dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Individual cellular responses to three different methods of inducing intracellular acidosis were studied: 1) pHₑ and pHᵢ were varied by hypercapnic acidification, 2) pHₑ was held constant, whereas intracellular acidosis was induced using the ammonia prepulse method, and 3) pHₑ was held constant, whereas intracellular acidosis was induced using isohydric hypercapnia. We also examined the pHᵢ regulatory mechanisms whereby neurons within the subesophageal ganglia responded to intracellular acidosis.

MATERIAL AND METHODS

H. aspersa were purchased throughout the year (Pennsylvania Snail) and maintained in a humidified aquarium at 22°C. The snails were fed carrots, lettuce, cucumbers, and cornmeal as previously described (11).

Solutions. Control saline consisted of (in mM) 85 NaCl, 4 KCl, 7 CaCl₂, 5 MgCl₂, buffered with 20 HEPES (HEPES free-acid; Sigma, St. Louis, MO) and titrated with NaOH to pH 7.8. The hypercapnic solutions contained (in mM) 20 NaHCO₃, 90 NaCl, 4 KCl, 7 CaCl₂, 5 MgCl₂, 0.2 NaH₄PO₄ equilibrated with CO₂ to pH 7.5 (5% CO₂) or 7.2 (10% CO₂). The ammonia prepulse solution contained (in mM) 10 NH₄Cl, 75 NaCl, 3 CaCl₂, 5 MgCl₂, 4 KCl, 0.2 NaH₄PO₄ buffered with 20 HEPES free-acid and titrated with NaOH to pH 7.8. Without HEPES in solution, we had persistent difficulties preventing CaCO₃ precipitation at room temperature even with added 0.2 NaH₂PO₄, just as Thomas described (27). In addition, we did not use control solutions for NH₄Cl perfusion in which Na⁺ was held constant by substituting N-methyl-d-glucamine. N-methyl-d-glucamine-containing solutions were also not stable at pH 7.8 and room temperature. The isohydric hypercapnic solution consisted of (in mM) 40 NaHCO₃, 60 NaCl, 7 CaCl₂, 5 MgCl₂, 4 KCl, 0.2 NaH₄PO₄ buffered with 20 HEPES free-acid and equilibrated with CO₂ to pH 7.8 (5% CO₂). In isohydric solutions containing inhibitors, amiloride (1 mM; Sigma) and DIDS (20 μM; Sigma) were used. The sodium-free BCECF calibration solution consisted of (in mM) 110 KCl, 7 CaCl₂, 5 MgCl₂ buffered with 10 HEPES free-acid and titrated with KOH to pH 7.2. The acetoxy-methyl ester of BCECF (Molecular Probes, Junction City, OR) was prepared as a 3.4-mM stock solution in DMSO (1 mg/500 μl) and diluted to 30 μl (35.2 μl/4 ml) in control saline. Nigericin (Molecular Probes) was prepared as a 27.5-mM stock solution in DMSO (10 mg/500 μl) and diluted to 16 μl (59.6 μl/100 ml) in the calibration solution. The osmolality of all the solutions was 225 ± 5 mosmol/kg H₂O.

Isolated central nervous system preparation. The subesophageal ganglia and the cerebral ganglia were removed after sectioning all neural connectives and the aorta as described previously (11). The isolated central nervous system was pinned with the dorsal surface exposed in a perfusion chamber contained within a petri dish. The subesophageal ganglia were covered by a thick outer sheath and a thin inner sheath lying directly on and within the neurons of the ganglia. The outer sheath was removed manually and the inner sheath was treated with protease (1 mg/ml; Sigma) for 8 min and delicately pulled away. The protease was rinsed from the chamber with repeated washings with control saline. The isolated central nervous system was incubated in control saline with 30 μM BCECF at room temperature (22°C) for 1.25 h in the dark. A coverslip was placed over the perfusion chamber to create a uniform plane of vision and to ensure even perfusion over the isolated central nervous system. The isolated central nervous system was washed with control saline for 10–15 min to remove any remaining extracellular BCECF. Test solutions perfused the bath via gravity-fed tubing at a rate of 10 ml/min. The perfusion chamber was relatively large, and complete solution changes required 30 s. A small pH electrode (“Beetroot,” World Precision Instruments, Sarasota, FL) was used to confirm that the effluent pH from the perfusion chamber was equivalent to the pH entering the chamber.

Imaging of BCECF-loaded neurons. After preparation, the dish was placed under an Optiphot-2 upright microscope (Nikon, Melville, NY) mounted with a SenSys charge-coupled device (CCD) camera (Photometrics, Tucson, AZ) connected to a Dimension XPS computer (Dell Computer, Austin, TX). Neurons on the subesophageal ganglia were excited for 300–500 ms with light from a 75-W xenon arc lamp (Interlight, Hammond, IA) that was filtered (440 and 550 nm) using a Lambda 10–2 filter wheel (Sutter Instrument, Novato, CA). Emitted light was captured by the CCD camera after passing through a dichroic mirror with a high pass cutoff of 515 nm and a 530 ± 15.5-nm emission filter (Chroma Technology, Brattleboro, VT). We used Axon Imaging Workbench software (Axon Instruments, Foster City, CA) to control the filter wheel and collect and process the data.

Calibration of pHᵢ from BCECF fluorescence. pHᵢ was measured from the ratio of BCECF-emitted fluorescence after excitation at 500 and 440 nm. A calibration curve of pHᵢ as a function of normalized fluorescence ratios (Nᵢ; normalized to pH 7.2) was calculated as described by Boyarsky et al. (4). Neurons were perfused with solutions of known pHᵢ ranging from 6.5 to 8.5, and pHᵢ values were measured after equilibration between pHₑ and pHᵢ using the high K⁻/nigericin technique. From calibration of pHᵢ as a function of Nᵢ, a calibration curve to transform Nᵢ ratios into pHᵢ was constructed using the following equation: pHᵢ = 7.2073 + log [(Nᵢ – 0.55378)/(1.45378 – Nᵢ)]; r² = 0.98; n = 67. A single-point calibration (pH 7.2, Nᵢ = 1.0) was performed at the conclusion of each experiment, and pHᵢ values were determined from the calibration curve.

pH response protocols. All experiments were conducted at room temperature (22°C). Only neurons in which BCECF
fluorescence at 440 nm diminished <0.5%/min over the course of an experiment were analyzed. BCECF is a vital dye, and a low leakage rate is an indicator of cell viability. Ritz et al. (23, 24) pointed out that pH* seemed to control the effectiveness of pH regulation. Therefore, we designed protocols to reduce pH* while pH was reduced or held constant. During hypercapnic acidoses, CO₃ readily penetrates the intracellular space and pH* and pH both fall. Two levels of hypercapnic acidoses were studied to establish a dose-response relationship, pH 7.5, 5% CO₂, and pH 7.2, 10% CO₂. During ammonia prepulse, pH* is held constant throughout the protocol, although pH falls after NH₄Cl is removed from the perfusate (3). We selected a concentration of NH₄Cl and an NH₄Cl perfusion time that generated an intracellular acidoses equivalent to the fall in pH associated with milder hypercapnic acidoses (pHe 7.5, CO₂ 5%). In the isohydric hypercapnic experiment, pH was constant and pH dropped. The extracellular HCO₃ concentration was raised to keep pH constant when the CO₂ was raised to 5%. However, CO₂ penetrated the cell and created an intracellular acidoses. In a final set of studies, the effects on pHₗ of ammonia regulation of amiloride (1 mM), DIDS (20 μM), and combined amiloride (1 mM) and DIDS (20 μM) were investigated after a rate of pH recovery had been established during perfusion with inhibitor-free isohydric hypercapnia.

Analysis and statistics. We wanted to compare the pattern of pHₗ regulation of individual neurons in the chemosensitive and nonchemosensitive areas during acidic stimulation. We measured pH in neurons from all ganglia on the dorsal surface of the subsophageal ganglia: the right and left parietal ganglia and the visceral ganglia. In each experiment, we chose the cells that had the best BCECF filling without regard to the location of the neurons. We defined the chemoreceptor region as the upper visceral, right visceral, and left parietal ganglia and the visceral ganglion. In each experiment, the CO₂ chemoreceptor region (stippled area) lies on either side of the cleft between the visceral and right parietal ganglia. The control pHi values and the pHₗ response of a single neuron from the chemoreceptor area is shown in Fig. 2. Each experiment consisted of two acidic stimuli (2 levels of hypercapnic acidosis, hypercapnic acidosis and ammonia pre-pulse, or hypercapnic acidosis and isohydric hypercapnia) and two levels of pHₗ were compared within each treatment: the initial, lowest pHₗ measured within 3 min of exposure to each treatment and a recovery pHₗ normalized to a constant duration of recovery (1 h). The actual recovery period was variable among neurons and usually lasted 15–25 min. We used a two-way ANOVA in which the region (chemoreceptor vs. nonchemoreceptor) was a between-subjects factor and type of acidic stimuli and pHₗ level (initial vs. recovery) were within-subjects factors. In the analysis of drug effects on pHₗ during isohydric hypercapnia, a similar ANOVA was used, but there were two between-subjects factors: region and drug treatment (amiloride, DIDS or combined amiloride, and DIDS). The within-subjects factors, type of treatment (isohydric hypercapnia with or without drug) and pHₗ level (initial vs. recovery), remained the same. When the results of an ANOVA indicated that significant differences existed among treatment conditions, specific preplanned comparisons were made after adjusting P values by the Bonferroni method to keep the overall P value in each experiment at P ≤ 0.05.

RESULTS

Hypercapnic acidosis. An example of the protocol and the pHₗ response of a single neuron from the chemoreceptor region on the dorsal surface of the subsophageal ganglia is shown in Fig. 2. Each experiment began with measurements of pHₗ during perfusion with control saline at pH 7.8 and no added CO₂. A pHₗ of 7.8 is within the normal range of hemolymph pH in intact, active snails (7). Two levels of hypercapnic acidosis were studied: pHₗ 7.2, 10% CO₂ and pHₗ 7.5, 5% CO₂ (the normal hemolymph CO₂ concentration is ~2.5%; Ref. 7). The order of testing pHₗ 7.2 and pH 7.5 was varied, but in the example shown in Fig. 2, the pHₗ 7.5, 5% CO₂ was studied first. The pHₗ fell quickly after each hypercapnic stimulation began, and pHₗ fell more when pHₗ was 7.2 compared with pHₗ 7.5. The rate of pHₗ recovery was not significantly different from zero at either level of hypercapnic acidosis.

We studied 20 cells within the chemoreceptor region and 19 cells outside the chemoreceptor region. The nonchemoreceptor cells were distributed equally over the dorsal surface to the right and left parietal ganglia and the visceral ganglia. The control pHₗ values and the initial pHₗ values immediately after the onset of acidic stimuli were applied are shown in Table 1. The
initial pH$_i$ values were the lowest pH$_i$ values measured within 3 min of applying the test solution. pH$_i$ fell progressively and significantly as the CO$_2$ in the perfusate was raised from 0 to 5 and 10% and the pH$_e$ fell from 7.8 to 7.5 and 7.2, respectively. However, the initial pH$_i$ values at pH$_e$ 7.8, 7.5, and 7.2 were not significantly different between chemoreceptor and nonchemoreceptor regions.

The average effects of two levels of hypercapnic acidosis in the chemoreceptor region and nonchemoreceptor regions are shown in Fig. 3. Despite equivalent reductions in initial pH$_i$ and pH$_e$ during the two levels of hypercapnic acidosis, the rates of pH$_i$ recovery in the chemoreceptor region and nonchemoreceptor regions were significantly different. In the chemoreceptor region, the pH$_i$ recovery rates at pH$_e$ 7.5 and 7.2 were 0.016 ± 0.068 pH units/h and −0.113 ± 0.061 pH units/h, respectively. pH$_i$ recovery rates were faster in the nonchemoreceptor regions at both pH$_e$ values: at pH$_e$ 7.5 and 7.2, pH$_i$ recovery rates were 0.279 ± 0.069 and 0.053 ± 0.063 pH units/h, respectively. Hence, pH$_i$ recovery was slower in the chemoreceptor region compared with the nonchemoreceptors at both pH$_e$ 7.5 and 7.2 (P < 0.01), and pH$_i$ recovery was slower in both chemoreceptor and nonchemoreceptor regions at pH$_e$ 7.2 compared with pH$_e$ 7.5 (P < 0.01). In the chemosensitive area, 6 of 20 neurons tested recovered at 5% CO$_2$ and 2 of 20 neurons recovered at 10% CO$_2$. In the nonchemosensitive area, 12 of 19 neurons tested recovered at 5% CO$_2$ and 10 of 19 neurons recovered at 10% CO$_2$. Hence, pH$_i$ recovery during hypercapnic acidosis was significantly more frequent among neurons from nonchemoreceptor areas compared with the chemoreceptor region (χ$^2 = 4.31; P < 0.04$ analyzing only the 5% treatment level). However, the patterns of pH$_i$ recovery were not perfectly segregated between chemoreceptor and nonchemoreceptor regions: small numbers of neurons within the chemoreceptor area demonstrated pH$_i$ recovery, and a larger number of neurons in nonchemoreceptor regions failed to manifest significant pH$_i$ recovery.

Table 1. Initial pH$_i$ values during hypercapnic acidosis, ammonia prepulse, and isohydric hypercapnic protocols

<table>
<thead>
<tr>
<th>Region</th>
<th>n</th>
<th>Control pH$_e$ 7.8, 0% CO$_2$</th>
<th>Acidosis pH$_e$ 7.5, 5% CO$_2$</th>
<th>Acidosis pH$_e$ 7.2, 10% CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemoreceptor</td>
<td>20</td>
<td>7.51 ± 0.17</td>
<td>7.19 ± 0.21</td>
<td>6.99 ± 0.22</td>
</tr>
<tr>
<td>Non-chemoreceptor</td>
<td>19</td>
<td>7.52 ± 0.18</td>
<td>7.20 ± 0.18</td>
<td>6.96 ± 0.17</td>
</tr>
</tbody>
</table>

**Ammonium chloride prepulse protocol.** After ammonia prepulse, pH$_i$ regulatory mechanisms were studied in chemoreceptor and nonchemoreceptor regions, whereas pH$_e$ changed, but pH$_i$ was held constant. An example of the progression of pH$_i$ during an ammonia prepulse experiment from a single neuron in the nonchemoreceptor part of the right parietal ganglion is shown in Fig. 4. Each experiment began with a control measurement of pH$_i$ at pH$_e$ 7.8. The pattern of pH$_i$ recovery during hypercapnic acidosis (pH$_e$ 7.5, 5% CO$_2$) was determined, and this was followed by NH$_4$Cl exposure (10 mM) at pH$_e$ equal to 7.8 for 10 min. After NH$_4$Cl was removed from the perfusate, pH$_e$ was kept at 7.8. In the cell shown in Fig. 4, pH$_i$ was 7.4 when pH$_e$ was 7.8 during the control period. This neuron demonstrated significant pH$_i$ recovery during hypercapnic acidosis (recovery rate equal 0.703 pH units/h; P < 0.001). When returned to pH$_e$ 7.8 and no CO$_2$, there was an alkaline overshoot, which was a further manifestation of pH$_i$ recovery during hypercapnic acidosis. During NH$_4$Cl perfusion, the cell was alkalized, but pH$_i$ fell once NH$_4$Cl was removed from the perfusate.
The rate of pH$_i$ recovery was greater after the ammonia prepulse perfusate. The pH$_i$ recovery values were calculated from the initial pH$_i$ and the recovery rate in each cell to estimate the pH$_i$ value that would have been present after 1 h of acidic stress. There were no differences among the initial pH$_i$ values between chemoreceptor and nonchemoreceptor regions. *pH$_i$ recovery rate of chemoreceptor and nonchemoreceptor regions were significantly lower at pH$_e$ 7.2 compared with pH$_e$ 7.5 ($P < 0.01$). #pH$_i$ recovery rates at both pH$_e$ 7.5 and 7.2 were significantly slower in the chemoreceptor region compared with nonchemoreceptor region neurons ($P < 0.01$).

The average responses in 9 neurons from the chemoreceptor area and 25 neurons from nonchemoreceptor areas are shown in Fig. 5. The control pH$_e$ values and the initial pH$_i$ values during hypercapnic acidosis and the acidification phase of the ammonia prepulse protocol were not significantly different between regions (see Table 1). However, the rates of pH$_i$ recovery were different between regions and between methods of acidification (Fig. 5). The rate of recovery was negligible, $-0.045 \pm 0.088$ pH units/h in the chemoreceptor region during hypercapnic acidosis. The recovery rate increased to $0.550 \pm 0.159$ pH units/h in the same neurons during ammonia prepulse acidification at pH$_e$ equal to 7.8. A similar change occurred in the nonchemoreceptor regions: pH$_i$ recovery was $0.262 \pm 0.053$ pH units/h during hypercapnic acidosis and increased to $0.737 \pm 0.096$ pH units/h during the ammonia prepulse acidification phase. The pattern of pH$_i$ recovery rate was similar in chemoreceptor neurons and nonchemoreceptor neurons; the slope of the pH$_i$ recovery was less in chemoreceptor region neurons during both treatment conditions (hypercapnic acidosis and after NH$_4$Cl), but the difference in slopes between chemoreceptor and nonchemoreceptor regions failed to reach statistical significance in the ANOVA ($P = 0.056$).

However, the pH$_i$ recovery rate was significantly greater during ammonia prepulse acidification compared with hypercapnic acidosis in both chemoreceptor and nonchemoreceptor regions ($P < 0.001$). Finally, the presence or absence of NaHCO$_3$ in the perfusate did not alter the rate of pH$_i$ recovery after the ammonia prepulse (data not shown).

**Isohydric hypercapnia.** Isohydric hypercapnia is an alternative mechanism to the ammonia prepulse protocol, whereby pH$_e$ remains constant while pH$_i$ is reduced. The increased CO$_2$ present during hypercapnia quickly diffuses into the neuron and acidifies the intracellular space, but the pH$_e$ is held constant because the increase in CO$_2$ in the extracellular fluid is matched by increased bicarbonate. This method has the further advantage that bicarbonate and CO$_2$ are present during the entire protocol. An example of this protocol and
the response of a single chemoreceptor neuron are shown in Fig. 6. pHᵢ in the neuron shown in Fig. 6 dropped from a control pHᵢ value of 7.4 when pHₑ was 7.8 to a pHᵢ of ~7.13 when pHₑ was 7.5. There was no evidence of recovery of pHᵢ during hypercapnic acidosis. During isohydric hypercapnia, pHᵢ did not fall quite as low (pHᵢ ~7.17) as it had when exposed to equivalent hypercapnia during the hypercapnic acidosis exposure, but pHᵢ recovered steadily during isohydric hypercapnia.

The average responses of 26 neurons in the chemoreceptor region and 14 neurons in nonchemoreceptor regions during hypercapnic acidosis (pHₑ 7.5, 5% CO₂) and isohydric isocapnia (pHₑ 7.8, 5% CO₂) are shown in Fig. 7. As in the previous experiments, the initial pHᵢ values in the control condition, hypercapnic acidosis, and isohydric hypercapnia were not significantly different between chemoreceptor region and nonchemoreceptor regions (see Table 1). Furthermore, the initial pHᵢ values during hypercapnic acidosis and isohydric hypercapnia were not significantly different from each other, but both values were significantly less than the control pHᵢ. The pHᵢ recovery rate was 0.002 ± 0.020 (pHₑ 7.5, 5% CO₂) and 0.267 ± 0.180 pH units/h (pHₑ 7.8, 5% CO₂) in the chemoreceptor region neurons. In neurons from nonchemosensitive areas, the pHᵢ recovery rate was 0.249 ± 0.188 (pHₑ 7.5, 5% CO₂) and 0.396 ± 0.115 pH units/h (pHₑ 7.8, 5% CO₂). The pHᵢ recovery rate was significantly less in chemoreceptor neurons during both hypercapnic acidosis and isohydric hypercapnia compared with nonchemoreceptor region neurons (P < 0.001). Furthermore, the pHᵢ recovery rate was slower during hypercapnic acidosis compared with isohydric hypercapnia in both chemoreceptor and nonchemoreceptor neurons (P < 0.001).

**Pharmacological studies of pHᵢ regulatory mechanisms.** Amiloride inhibits Na⁺/H⁺ exchange (2), and DIDS is a chloride channel inhibitor that blocks Cl⁻/HCO₃⁻ exchange (8). We studied the effect of both drugs on the rate of pHᵢ recovery in neurons in the chemoreceptor region and nonchemoreceptor regions. After stabilization of pHᵢ in control saline (pHₑ 7.8), the neurons were exposed to hypercapnic and acidic saline (pHₑ 7.5, 5% CO₂) to determine the pattern of pHᵢ regulation when both pHₑ and pHᵢ were changed. Subsequently, each neuron was also exposed to isohydric hypercapnia (pHₑ 7.8, 5% CO₂) or isohydric hypercapnia with amiloride (1 mM) or DIDS (20 μM) and amiloride (1 mM) and DIDS (20 μM). This concentration of DIDS was selected because it modified pneumostomal activity in previous studies (10) and comparable concentrations of SITS inhibited Na⁺/HCO₃⁻ exchange in H. aspersa (29, 30). The order of these treatments (isohydric hypercapnia with or without drug) was varied. We studied the drug effects during isohydric hypercapnia to increase the number of neurons with significant rates of pHᵢ recovery, and we analyzed only neurons that demonstrated a significant rate of pHᵢ recovery in the absence of drug treatment. We made this selection to avoid difficulties determining whether amiloride and DIDS altered the rate of pHᵢ recovery in neurons with extremely slow rates of recovery. Of 34 neurons studied in the chemoreceptor region, 8 were excluded, and of 19 neurons from nonchemoreceptor regions, 3 were excluded on the basis of slow rates of pHᵢ recovery. The rates of recovery were less in chemoreceptor region neurons compared with neurons from nonchemoreceptor regions, but the pattern of responses to amiloride and DIDS was not significantly different between regions. Therefore, the data from all regions were combined, and the pattern of pHᵢ recovery during hypercapnic acidosis was dropped from the analysis of drug effects. The average responses of the neurons analyzed are shown in Fig. 8. The initial pHᵢ values during isohydric hypercapnia with and without drug treatment were significantly less in chemoreceptor region neurons compared with nonchemoreceptor region neurons (P < 0.001).
not different among treatment groups. Furthermore, the rates of pH\textsubscript{i} recovery in the absence of the particular drug treatment were not different among drug treatment groups. The rate of pH\textsubscript{i} recovery during exposure to DIDS (0.358 ± 0.159 pH units/h) was not different from the pH\textsubscript{i} recovery rate during the control isohydric hypercapnic exposure without DIDS (0.325 ± 0.125 pH units/h). Amiloride, however, caused a significant decrease in pH\textsubscript{i} recovery rate (−0.355 ± 0.342 pH units/h) compared with the control rate (0.336 ± 0.177 pH units/h; P < 0.05) in the same neurons and compared with the DIDS-treated neurons (P < 0.05). Amiloride plus DIDS caused a further significant drop in pH\textsubscript{i} recovery rates (−0.757 ± 0.418 pH units/h) compared with the control recovery rate in the same neurons (0.269 ± 0.212 pH units/h; P < 0.05). The pH\textsubscript{i} recovery rate during amiloride plus DIDS was also significantly less than the recovery rate with amiloride alone (P < 0.05). We repeated this analysis on the neurons in the chemoreceptor region alone, and the results were identical: no effect of DIDS alone, reduced recovery rates after treatment with amiloride, and a greater reduction in pH\textsubscript{i} recovery rates after treatment with amiloride and DIDS.

**DISCUSSION**

We compared pH\textsubscript{i} regulation in neurons within the CO\textsubscript{2} chemoreceptor region to pH\textsubscript{i} regulation in nonchemoreceptor regions on the dorsal surface of the subesophageal ganglia of *H. aspersa*. We biased the experiment toward finding no differences between areas by broadly defining the chemoreceptor region as the upper and right quadrant of the visceral ganglion and left quadrant of the right parietal ganglion. Nonchemoreceptor cells were defined as neurons in all other regions on the dorsal surface of the subesophageal ganglia. Despite this generous definition of the chemoreceptor regions, neurons in the chemoreceptor region were, on average, less able to regulate pH\textsubscript{i} under all acidic stimuli tested, although the initial pH\textsubscript{i} in all conditions tested was similar among chemoreceptor and nonchemoreceptor areas. Furthermore, pH\textsubscript{i} regulation was less effective in all neurons when the acidic stimulus was associated with a drop in pH\textsubscript{e}. The dominant pH\textsubscript{i} regulatory mechanism is probably an amiloride-sensitive Na\textsuperscript{+}/H\textsuperscript{+} exchanger, but there may be a small role for a DIDS-sensitive Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange mechanism. These results are, in general, strikingly similar to results from neurons in CO\textsubscript{2}-sensitive regions in the brain stem of preweanling rats (23, 24) despite the independent evolution of aerial respiration in vertebrates and invertebrates.

**Control and initial pH\textsubscript{i} values.** The pH\textsubscript{i} of chemosensitive and nonchemosensitive neurons was not significantly different under steady-state conditions (control saline, pH 7.8) in any of our experiments. The resting steady state pH\textsubscript{i} varied between 7.4 and 7.5. This value is similar to pH\textsubscript{i} values (7.41 ± 0.08; mean ± SD) described previously by Thomas (28) using intracellular pH electrodes in neurons in *H. aspersa*. We studied three acidic stimuli: hypercapnic acidosis, ammonia prepulse, and isohydric acidosis, and in all cases, the initial pH\textsubscript{i} values measured within 3 min of applying each stimulus were similar among neurons from the chemoreceptor region and nonchemoreceptor regions. Therefore, any differences in pH\textsubscript{i} recovery (see below) cannot be attributed to differences in the initial intracellular or extracellular pH. However, the lack of differences in pH\textsubscript{i} among chemoreceptor and nonchemoreceptor regions during acidic stimulation is at odds with some previous work. When pH\textsubscript{e} was changed from 7.48 to 7.30 in the experiments described by Ritucci et al. (24), pH\textsubscript{i} fell by ~83% of the fall in pH\textsubscript{e} in the NTS; the reduction in pH\textsubscript{i} was ~33% of the fall in pH\textsubscript{e} in the VLM. In contrast, pH\textsubscript{i} fell by only 4–22% of the change in pH\textsubscript{e} in nonchemosensitive regions (the inferior olive and hypoglossal nucleus). In isolated glomus cells of the rabbit carotid body, which are CO\textsubscript{2} sensitive, pH\textsubscript{i} fell by ~60–70% of the change in pH\textsubscript{e} during hypercapnic acidosis (5). The usual change in pH\textsubscript{i} is ~20–30% of the change in pH\textsubscript{e} in other nonchemosensitive tissues (see Ref. 5 for a complete list of references). In the snail neurons, the change in pH\textsubscript{i} was 71% of the change in pH\textsubscript{e} when pH\textsubscript{e} changed from 7.5 to 7.2 during hypercapnic acidosis, comparable to the results in the NTS and in carotid body glomus cells. However, the change in pH\textsubscript{i} for this change in pH\textsubscript{e} was not different between chemoreceptor and nonchemoreceptor cells.

The rate of pH\textsubscript{i} recovery was slow (see below), and we estimated the buffering capacity in these neurons without including drugs to inhibit proton or bicarbonate exchange. We calculated the changes in intracellular
lar HCO₃⁻ associated with the measured changes in pH, apparent within 3 min of changing from control saline (pH 7.8, nominally CO₂ free) to either pH 7.5 (5% CO₂, 20 mM NaHCO₃) or pH 7.2 (10% CO₂, 20 mM NaHCO₃) using an apparent pKa of carbonic acid and a CO₂ solubility coefficient derived from pulmonate snail hemolymph (1). The estimated buffering capacity at pH 7.5 was 17.5 ± 11.5 and 50.7 ± 25.7 meq H⁺/pH unit at pH 7.2. These values are similar to those described by Thomas (27) in nonchemosensitive neurons using the same method. The particular value of the buffering capacity is of less interest in our study, however, than the lack of any difference in buffering capacity between chemoreceptor and nonchemoreceptor regions. Buckler et al. (5) indicated that a steep pHᵢ vs. pHₑ relationship was present in cells that acted as sensitive pH detectors. However, we found no such relationship in the neurons we studied: neurons in nonchemoreceptor areas had pHᵢ vs. pHₑ relationships as steep as neurons in the chemoreceptor region. The nonchemoreceptor regions might have some nonrespiratory chemoreceptor function, but the ubiquity of the steep pHᵢ vs. pHₑ relationship in the neurons that we studied leads us to conclude that a steep pHᵢ vs. pHₑ relationship is a necessary, but not sufficient, marker of pH sensitivity.

Intracellular pH regulation. Three main points emerge from the studies of the rate of pHᵢ regulation. First, regulation of pHᵢ during acidic stress was slower and less effective in neurons from the chemoreceptor region in all conditions studied. Second, the rate of pHᵢ regulation was slower in all regions when pHₑ was reduced compared with acidic stresses of equal intracellular severity but constant pHₑ. Finally, the inhibitory effect of pHₑ on the rate of pHᵢ recovery was graded: the lower the pHₑ, the slower the rate of pHᵢ recovery. The actual rates of pHᵢ recovery that we observed were similar to those reported by Thomas (30) in Helix aspersa, but slightly slower than the recovery rates reported by Ritucci et al. (24) in rat neurons studied at 37°C. During hypercapnic acidosis at both pHₑ 7.5 and 7.2, pHᵢ regulation was significantly slower in neurons in the chemoreceptor region compared with nonchemoreceptor regions. The responses to hypercapnic acidosis were consistent with the hypothesis that CO₂ chemoreceptors should exhibit reduced or no pHᵢ recovery in response to CO₂-induced cellular acidification just as Ritucci et al. (24) found in rat brain stem slices and Buckler et al. (5, 6) found in isolated type I carotid body cells.

The lack of pHᵢ regulation could be due to the absence of pHᵢ recovery mechanisms in neurons from the chemosensitive area or the inhibition of pHᵢ regulation during hypercapnic acidosis. To investigate whether chemoreceptor region neurons simply lack effective pHᵢ recovery mechanisms, we acidified the neurons while maintaining pHₑ constant using an ammonia prepulse protocol. The rate of pHᵢ recovery in chemoreceptor region neurons was still slower than recovery in nonchemoreceptor regions, but pHᵢ recovery within the chemoreceptor region was much faster when pHₑ was equal to the control pHₑ than recovery during hypercapnic acidosis. Hence, neurons within the chemoreceptor region possess pHᵢ regulatory mechanisms, but the mechanisms were inhibited by hypercapnic acidosis. Therefore, we tried to determine whether the lack of pHᵢ regulation was the result of the hypercapnia or the extracellular acidosis. Regulation of pHᵢ during isohydric hypercapnia (5% CO₂) was more rapid than pHᵢ regulation during hypercapnic acidosis (5% CO₂), from which we infer that pHᵢ inhibited pHᵢ regulation in neurons within the chemoreceptor region. The pHᵢ recovery mechanisms of a variety of cell types are inhibited by a decrease in pHₑ (22). Among the conditions we studied, pHᵢ recovery was faster when pHₑ was held constant. Neurons in the chemoreceptor region had a slower rate of pHᵢ recovery compared with neurons from the nonchemoreceptor regions whether pHᵢ and pHₑ changed (hypercapnic acidosis) or only pHᵢ changed (ammonia prepulse and isohydric hypercapnia). We infer from the reduced rates of pHᵢ recovery during ammonia prepulse and isohydric hypercapnia that the capacity for pHᵢ regulation was reduced in the chemoreceptor region even at the control pHₑ (7.8). The rate of pHᵢ recovery was further reduced when pHₑ was also reduced.

pHᵢ regulatory mechanisms in chemoreceptor and nonchemoreceptor neurons. We examined the type of pHᵢ regulatory mechanisms present in neurons within the chemoreceptor region compared with nonchemoreceptor regions. We used amiloride to inhibit Na⁺/H⁺ exchange and DIDS to inhibit Cl⁻-dependent HCO₃⁻ exchange. The pattern of inhibition of pHᵢ regulation did not differ significantly between chemosensitive and nonchemosensitive neurons when tested with amiloride and/or DIDS during isohydric hypercapnia. Hence, we found no evidence that different pHᵢ regulatory mechanisms were present in neurons within the chemosensitive region compared with nonchemosensitive regions. When DIDS alone was applied, the rate of pHᵢ recovery during isohydric hypercapnia and DIDS administration was equivalent to the rate of pHᵢ recovery when neurons were perfused with an inhibitor-free isohydric hypercapnic solution. When amiloride was applied, the rate of recovery was significantly slowed. Thus the dominant pHᵢ regulatory mechanism in both chemoreceptor and nonchemoreceptor regions seems to be an Na⁺/H⁺ exchanger. However, when both amiloride and DIDS were applied, the rate of pHᵢ recovery was further reduced below the rate of recovery during perfusion with amiloride alone. The data suggest that Cl⁻-dependent HCO₃⁻ exchange may also regulate pHᵢ, but the Na⁺/H⁺ exchanger suffices to regulate pHᵢ when Cl⁻-dependent HCO₃⁻ exchange is inhibited. Thomas (29, 30) put forward the idea that Na⁺-dependent Cl⁻/HCO₃⁻ exchange was the essential pHᵢ-regulating transporter in Helix neurons, but recently Thomas (31) also found evidence of an Na⁺/H⁺ exchange mechanism in Helix neurons. Hence, it seems likely that pHᵢ regulatory mechanisms are more heterogeneous in Helix than was first appreciated.
Once again, the results of the studies are remarkably similar to the pH regulatory processes described in brain stem slice preparations from chemoreceptor and nonchemoreceptor regions in rats (24, 25). An Na+/H+ exchanger in rat medullary slices was the only pH-regulating transporter activated during acidosis in NTS and VLM neurons (chemosensitive regions). Ritucci et al. (23) demonstrated that Na+/H+ exchange mechanisms were present in both chemoreceptor and nonchemoreceptor regions, but the pH of half-maximal inhibition of Na+/H+ exchange was significantly higher in chemoreceptor regions. Our findings in *Helix* are consistent with this hypothesis: one need not posit different pH regulatory mechanisms, the results may be consistent with greater inhibition of Na+/H+ exchange by pH inhibition of Na+/H+ exchange that may be present in chemoreceptor regions even at normal, control pH values. However, the results in *Helix* are susceptible to another interpretation. pH recovery was slower in the chemoreceptor region compared with nonchemoreceptor regions under all conditions. Therefore, neurons within the chemoreceptor region may have an absolute reduction in pH regulatory capacity (e.g., less expression of the Na+/H+ exchanger per neuron).

**Perspectives**

*pH* regulation and ventilatory control. In our previous electrophysiological studies of CO2-chemosensitive neurons, we found very few intrinsically CO2-sensitive neurons in each chemosensitive area, perhaps 8–12 neurons (14). It was our expectation that poor pH regulation might be present only in a few cells in the chemosensitive area of *H. aspersa*, and we did find that poor pH regulation among neurons in the subesophageal ganglia was significantly (*P* = 0.007) segregated and much more likely in neurons confined to the CO2-sensitive area. However, the segregation was not perfect; many neurons outside the CO2-sensitive area demonstrated poor pH recovery during hypercapnic acidosis. Ritucci et al. (24) also expected only 30–40% of the neurons in chemosensitive regions to show delayed or reduced pH recovery during hypercapnia, but found poor pH recovery in the majority of cells in chemosensitive areas. In the NTS, 36 of 39 neurons did not recover; in the VLM, 33 of 38 neurons did not recover. These findings contrast with prompt pH recovery in 100% of nonchemosensitive neurons in the rat brain stem. The implication of these results is that identification of neurons as chemosensitive based on pH regulatory profiles will be an insensitive marker of chemosensitivity: poor pH regulation is ubiquitous, electrophysiological evidence of CO2 chemosensitivity is more circumscribed. We conclude that a delayed or flat pH recovery profile during intracellular and extracellular acidification is a necessary, but not sufficient, condition for CO2 chemoreceptor neurons in both molluscan and murine preparations.

If neurons with flat pH regulatory profiles during hypercapnic acidosis play an important role in CO2 chemosensory regulation of ventilation (and that is certainly our hypothesis), then the whole animal ventilatory responses to manipulations of pH should correlate well with the single chemoreceptor neuron response. This is not, however, uniformly the case. Perfusion of the brain stem of awake rabbits with artificial cerebrospinal fluid containing 10 μM DIDS did not change resting ventilation, but did increase the ventilatory response to CO2 (20). These results imply that DIDS reduced the pH in or about chemoreceptor cells. However, DIDS had no effect on pH or pH regulation during hypercapnic acidosis in the rat brain stem (24). Similar problems of interpretation exist in snails. DIDS increased normocapnic pneumostomal activity in *H. aspersa* (10), but DIDS alone did not alter pH or pH regulation in neurons within the chemoreceptor area. Amiloride (1 mM) administered via cisternal perfusion to anesthetized rabbits increased minute ventilation under control conditions, but did not alter ventilatory sensitivity to CO2 (21). The effects of amiloride on pH and pH regulation in chemoreceptor area neurons in the rat brain stem are the exact opposite of those expected: amiloride did not change pH under control, normocapnic conditions but did reduce pH regulation during hypercapnia (24). We have not yet tested the effect of amiloride on pneumostomal activity. The divergence between whole animal ventilatory responses and chemoreceptor area pH responses is not irreconcilable. For example, the DIDS effects in the whole animal might reflect changes in pH regulation originating in nonchemoreceptor areas that nonetheless alter pH in chemoreceptor areas and thereby modify chemoreceptor activity. The responses may also originate from non-acid-base effects of the drugs that obscure the drug effects on pH. For example, amiloride caused marked generalized excitation in awake rabbits during cisternal perfusion (21). Finally, there is, as yet, no electrophysiological proof that neurons in the NTS and rostral ventrolateral medulla of rats and in the chemosensitive area of *Helix*, in which pH regulation is poor, are actually CO2 chemosensors, although that is our working hypothesis. Nonetheless, the lack of correlation between pharmacological manipulation of the whole animal ventilatory responses to CO2 and single-neuron pH regulation is disconcerting for any theory of respiratory control that posits a key role for pH in chemoreceptor areas.

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