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

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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 pHᵢ-pHe 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, pulmonary, 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 pHᵢ-pHe 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...
nonchemosensitive areas, the inferior olive and hypoglossal 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 recovered 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) pHᵢ 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 NaH₂PO₄ equilibrated with CO₂ to pH 7.5 (5% CO₂) or 7.2 (10% CO₂). The ammonia prepulse solution consisted (in mM) 10 NH₄Cl, 75 NaCl, 7 CaCl₂, 5 MgCl₂, 4 KCl, 0.2 NaH₂PO₄ 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).

**Calibration process.** 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 acetoxymethyl 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 μM (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 μM (59.6 μl/100 ml) in the calibration solution. The osmolality of all the solutions was 225 ± 5 mosmol/kgH₂O.

**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 preparation with control saline, 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 (“Beetrode,” 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, LA) that was filtered (440 and 500 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 ± 12.5-nm emission filter (Chroma Technology, Brattleboro, VT). We used Axon Imaging Workbench (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 (Nₑ/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 Nₑ/Nᵢ, a calibration curve to transform Nₑ ratios into pHᵢ was constructed using the following equation: pHᵢ = 7.2073 + log [(Nₑ - 0.55378)/(1.45378 - Nᵢ)]; r² = 0.98; n = 67. A single-point calibration (pH 7.2, Nₑ = 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. Ritucci et al. (23, 24) pointed out that pHi seemed to control the effectiveness of pH regulation. Therefore, we designed protocols to reduce pHi while pH was reduced or held constant. During hypercapnic acidosis, CO2 readily penetrates the intracellular space and pHi and pH both fall. Two levels of hypercapnic acidosis were studied to establish a dose-response relationship, pH 7.5, 5% CO2, and pH 7.2, 10% CO2. During ammonia prepulse, pHi 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 acidosis equivalent to the fall in pHi associated with milder hypercapnic acidosis (pHe 7.5, CO2 5%). In the isohydric hypercapnic experiment, pHi 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 acidosis. In a final set of studies, the effects on pHi regulation of amiloride (1 mM), DIDS (20 μM), and combined amiloride (1 mM) and DIDS (20 μM) were investigated after a rate of pHi recovery had been established during perfusion with inhibitor-free isohydric hypercapnia.

Analysis and statistics. We wanted to compare the pattern of pHi regulation of individual neurons in the chemosensitive and nonchemosensitive areas during acidic stimulation. We measured pHi in neurons from all ganglia on the dorsal surface of the subesophageal ganglia: the right and left parietal ganglia and the visceral ganglion. 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 visceral ganglia and the visceral ganglion. In each experiment, 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 acidosis. In a final set of studies, the effects on pHi regulation of amiloride (1 mM), DIDS (20 μM), and combined amiloride (1 mM) and DIDS (20 μM) were investigated after a rate of pHi recovery had been established during perfusion with inhibitor-free isohydric hypercapnia.

Hypercapnic acidosis. An example of the protocol and the pHi response of a single neuron from the chemoreceptor region on the dorsal surface of the subesophageal ganglia is shown in Fig. 2. Each experiment began with measurements of pHi during perfusion with control saline at pH 7.8 and no added CO2. A pHi of 7.8 is within the normal range of hemolymph pH in intact, active snails (7). Two levels of hypercapnic acidosis were studied: pHi 7.2, 10% CO2 and pHi 7.5, 5% CO2 (the normal hemolymph CO2 concentration is ~2.5%; Ref. 7). The order of testing pHi 7.2 and pH 7.5 was varied, but in the example shown in Fig. 2, the pHi 7.5, 5% CO2 was studied first. The pHi fell quickly after each hypercapnic stimulation began, and pHi fell more when pHe was 7.2 compared with pHe 7.5. The rate of pHi 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 ganglion. The control pHi values and the initial pHi values immediately after the onset of acidic stimuli were applied are shown in Table 1. The
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$_i$ regulation during hypercapnic acidosis (pH$_e$ 7.5, 5% CO$_2$) was determined, and this was followed by NH$_4$Cl exposure (10 mM) at pHe 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 (recovary 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

![Graph](http://ajpregu.physiology.org/)

Fig. 2. The pH$_i$ response of a single neuron in the chemoreceptor region is shown. The neuron was exposed to 2 levels of hypercapnic acidosis [extracellular pH (pHe) 7.5, 5% CO$_2$ and pHe 7.2, 10% CO$_2$]. During hypercapnic acidosis (a and b), there was no pH$_i$ recovery. There was also no significant alkaline overshoot (c) when the neuron was returned to the control perfusate (pHe 7.8, 0% CO$_2$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Aciddosis</th>
<th>Post-NH$_4$Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region</td>
<td>pH$_e$ 7.8, 0% CO$_2$</td>
<td>pH$_e$ 7.5, 5% CO$_2$</td>
<td>pH$_e$ 7.8, 0% CO$_2$</td>
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<tr>
<td>Chemoreceptor</td>
<td>7.51 ± 0.17</td>
<td>7.19 ± 0.21</td>
<td>6.99 ± 0.22</td>
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<td>Non-chemoreceptor</td>
<td>7.52 ± 0.18</td>
<td>7.20 ± 0.18</td>
<td>6.96 ± 0.17</td>
</tr>
<tr>
<td>Initial pH$_i$ value</td>
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<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Region</th>
<th>pH$_e$ 7.8, 0% CO$_2$</th>
<th>pH$_e$ 7.5, 5% CO$_2$</th>
<th>pH$_e$ 7.8, 0% CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>pH$_e$ 7.8, 0% CO$_2$</td>
<td>pH$_e$ 7.5, 5% CO$_2$</td>
<td>pH$_e$ 7.8, 0% CO$_2$</td>
</tr>
<tr>
<td>pH$_e$ 7.8</td>
<td>7.55 ± 0.16</td>
<td>7.15 ± 0.14</td>
<td>7.24 ± 0.17</td>
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<tr>
<td>pH$_e$ 7.5</td>
<td>7.50 ± 0.16</td>
<td>7.09 ± 0.19</td>
<td>7.07 ± 0.16</td>
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<tr>
<td>NH$_4$Cl</td>
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<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.05$</td>
</tr>
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</table>

### Isohydric hypercapnic protocol

<table>
<thead>
<tr>
<th>Region</th>
<th>pH$_e$ 7.8, 0% CO$_2$</th>
<th>pH$_e$ 7.5, 5% CO$_2$</th>
<th>pH$_e$ 7.8, 0% CO$_2$</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>pH$_e$ 7.8, 0% CO$_2$</td>
<td>pH$_e$ 7.5, 5% CO$_2$</td>
<td>pH$_e$ 7.8, 0% CO$_2$</td>
</tr>
<tr>
<td>pH$_e$ 7.8</td>
<td>7.40 ± 0.04</td>
<td>7.11 ± 0.06</td>
<td>7.15 ± 0.08</td>
</tr>
<tr>
<td>pH$_e$ 7.5</td>
<td>7.41 ± 0.06</td>
<td>7.13 ± 0.06</td>
<td>7.17 ± 0.09</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD; $n$ indicates the number of neurons studied. There were no differences in any of the initial pH$_i$ values between chemoreceptor and non-chemoreceptor neurons under any of the conditions. $P$ values are reported for comparisons of pooled chemoreceptor and nonchemoreceptor groups among treatment conditions. NS, no significant difference between treatment groups; pH$_e$, extracellular pH.
The rate of pH\textsubscript{i} recovery was greater after the ammonia prepulse perfusate. The pH\textsubscript{i} recovery values were calculated from the initial pH\textsubscript{i}, pHi rose initially, but fell as NH\textsubscript{4}Cl entered the cell (plateau acidification). After NH\textsubscript{4}Cl was removed from the perfusate, pH\textsubscript{i} fell initially, but rose as the cell responded to the intracellular acidosis (e). There was significant pH\textsubscript{i} recovery, and the rate of pH\textsubscript{i} recovery was greater after the ammonia prepulse than during hypercapnic acidosis.

Isohydric hypercapnia. Isohydric hypercapnia is an alternative mechanism to the ammonia prepulse protocol, whereby pH\textsubscript{i} remains constant while pH\textsubscript{e} is reduced. The increased CO\textsubscript{2} present during hypercapnia quickly diffuses into the neuron and acidifies the intracellular space, but the pH\textsubscript{e} is held constant because the increase in CO\textsubscript{2} in the extracellular fluid is matched by increased bicarbonate. This method has the further advantage that bicarbonate and CO\textsubscript{2} are present during the entire protocol. An example of this protocol and perfusate and pH\textsubscript{e} started to return toward the control pH\textsubscript{e} value almost immediately.

The average responses in 9 neurons from the chemoreceptor area and 25 neurons from nonchemoreceptor areas are shown in Fig. 5. The control pH\textsubscript{i} values and the initial pH\textsubscript{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\textsubscript{i} recovery were different between regions and between methods of acidification (Fig. 5). The rate of recovery was negligible, −0.045 ± 0.088 pH units/h, in the chemoreceptor region during hypercapnic acidosis. The recovery rate increased to 0.550 ± 0.159 pH units/h in the same neurons during ammonia prepulse acidification at pH\textsubscript{e} equal to 7.8. A similar change occurred in the nonchemoreceptor regions: pH\textsubscript{i} recovery was 0.262 ± 0.053 pH units/h during hypercapnic acidosis and increased to 0.737 ± 0.096 pH units/h during the ammonia prepulse acidification phase. The pattern of pH\textsubscript{i} recovery rate was similar in chemoreceptor neurons and nonchemoreceptor neurons; the slope of the pH\textsubscript{i} recovery was less in chemoreceptor region neurons during both treatment conditions (hypercapnic acidosis and after NH\textsubscript{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\textsubscript{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\textsubscript{3} in the perfusate did not alter the rate of pH\textsubscript{i} recovery after the ammonia prepulse (data not shown).

![Fig. 3. pH\textsubscript{i} values (means ± SD) have been plotted as a function of treatment condition for neurons on the dorsal surface of the subesophageal ganglia within the chemoreceptor region (●) and outside the chemoreceptor region (○). The initial pH\textsubscript{i} values in each condition were the lowest pH\textsubscript{i} values measured within 3 min of changing the perfusate. The pH\textsubscript{i} recovery values were calculated from the initial pH\textsubscript{i} and the recovery rate in each cell to estimate the pH\textsubscript{i} value that would have been present after 1 h of acidic stress. There were no differences among the initial pH\textsubscript{i} values between chemoreceptor and nonchemoreceptor regions. *pHi recovery rate of chemoreceptor and nonchemoreceptor regions were significantly lower at pH\textsubscript{e} 7.2 compared with pH\textsubscript{e} 7.5 (P < 0.01). #pHi recovery rates at both pH\textsubscript{e} 7.5 and 7.2 were significantly slower in the chemoreceptor region compared with nonchemoreceptor region neurons (P < 0.01).](Image 354x160 to 534x294)

![Fig. 4. The pH\textsubscript{e} response of a single neuron in a nonchemoreceptor region is shown. After a brief control period (a) during which pH\textsubscript{e} was stabilized at a pH\textsubscript{e} of 7.8 without CO\textsubscript{2}, the neuron was exposed to hypercapnic acidosis (pH\textsubscript{e} 7.5 and 5% CO\textsubscript{2}) and an ammonia prepulse protocol. During hypercapnic acidosis (b), there was significant pH\textsubscript{e} recovery. There was a significant alkaline overshoot (c) when the neuron was returned to the control perfusate (pH\textsubscript{e} 7.8, 0% CO\textsubscript{2}). During the ammonia prepulse (d), pH\textsubscript{e} rose initially, but fell as NH\textsubscript{4}Cl entered the cell (plateau acidification). After NH\textsubscript{4}Cl was removed from the perfusate, pH\textsubscript{e} fell initially, but rose as the cell responded to the intracellular acidosis (e). There was significant pH\textsubscript{e} recovery, and the rate of pH\textsubscript{e} recovery was greater after the ammonia prepulse than during hypercapnic acidosis.](Image 54x178 to 306x332)

![Fig. 5. pH\textsubscript{i} values (means ± SD) have been plotted as a function of treatment condition for neurons on the dorsal surface of the subesophageal ganglia within the chemoreceptor region (●) and outside the chemoreceptor region (○). There were no differences among the initial pH\textsubscript{i} values between chemoreceptor and nonchemoreceptor regions. *pHi recovery rate of chemoreceptor and nonchemoreceptor regions were significantly lower at pH\textsubscript{e} 7.2 compared with pH\textsubscript{e} 7.5 (P < 0.001).](Image 58x575 to 282x725)
the response of a single chemoreceptor neuron are shown in Fig. 6. pH$_i$ in the neuron shown in Fig. 6 dropped from a control pH$_i$ value of 7.4 when pH$_e$ was 7.8 to a pH$_i$ of $\sim$7.13 when pH$_e$ was 7.5. There was no evidence of recovery of pH$_i$ during hypercapnic acidosis. During isohydric hypercapnia, pH$_i$ did not fall quite as low (pH$_i$ $\sim$7.17) as it had when exposed to equivalent hypercapnia during the hypercapnic acidois exposure, but pH$_i$ recovered steadily during isohydric hypercapnia.

The average responses of 26 neurons in the chemoreceptor region and 14 neurons in nonchemoreceptor regions during hypercapnic acidosis (pH$_e$ 7.5, 5% CO$_2$) and isohydric isocapnia (pH$_e$ 7.8, 5% CO$_2$) are shown in Fig. 7. As in the previous experiments, the initial pH$_i$ 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 pH$_i$ values during hypercapnic acidosis and isohydric hypercapnia were not significantly different from each other, but both values were significantly less than the control pH$_i$. The pH$_i$ recovery rate was 0.002 ± 0.202 (pH 7.5, 5% CO$_2$) and 0.267 ± 0.180 pH units/h (pH$_e$ 7.8, 5% CO$_2$) in the chemoreceptor neuron regions. In neurons from nonchemosensitive areas, the pH$_i$ recovery rate was 0.249 ± 0.188 (pH$_e$ 7.5, 5% CO$_2$) and 0.396 ± 0.115 pH units/h (pH$_e$ 7.8, 5% CO$_2$). The pH$_i$ 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 pH$_i$ recovery rate was slower during hypercapnic acidosis compared with isohydric hypercapnia in both chemoreceptor and nonchemoreceptor neurons (P < 0.001).

**Pharmacological studies of pH$_i$ regulatory mechanisms.** Amiloride inhibits Na$^+$/H$^+$ exchange (2), and DIDS is a chloride channel inhibitor that blocks Cl$^-$ dependent HCO$_3^-$ exchange (8). We studied the effect of both drugs on the rate of pH$_i$ recovery in neurons in the chemoreceptor region and nonchemoreceptor regions. After stabilization of pH$_i$ in control saline (pH$_e$ 7.8), the neurons were exposed to hypercapnic and acidic saline (pH$_e$ 7.5, 5% CO$_2$) to determine the pattern of pH$_i$ regulation when both pH$_e$ and pH$_i$ were changed. Subsequently, each neuron was also exposed to isohydric hypercapnia (pH$_e$ 7.8, 5% CO$_2$) or isohydric hypercapnia with amiloride (1 mM) or DIDS (20 $\mu$M) or both amiloride (1 mM) and DIDS (20 $\mu$M). This concentration of DIDS was selected because it modified pneumostomal activity in previous studies (10) and comparable concentrations of SITS inhibited Na$^+$-dependent Cl$^-$/HCO$_3^-$ 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 pH$_i$ recovery, and we analyzed only neurons that demonstrated a significant rate of pH$_i$ recovery in the absence of drug treatment. We made this selection to avoid difficulties determining whether amiloride and DIDS altered the rate of pH$_i$ 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 pH$_i$ 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 pH$_i$ 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 pH$_i$ values during isohydric hypercapnia with and without drug treatment were...
not different among treatment groups. Furthermore, the rates of pHi recovery in the absence of the particular drug treatment were not different among drug treatment groups. The rate of pHi recovery during exposure to DIDS (0.358 ± 0.159 pH units/h) was not different from the pHi recovery rate during the control isohydric hypercapnia exposure without DIDS (0.325 ± 0.125 pH units/h). Amiloride, however, caused a significant decrease in pHi 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 pHi 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 pHi 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 pHi recovery rates after treatment with amiloride and DIDS.

**DISCUSSION**

We compared pHi regulation in neurons within the CO₂ chemoreceptor region to pHi 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 pHi under all acidic stimuli tested, although the initial pHi in all conditions tested was similar among chemoreceptor and nonchemoreceptor areas. Furthermore, pHi regulation was less effective in all neurons when the acidic stimulus was associated with a drop in pHe. The dominant pHi regulatory mechanism is probably an amiloride-sensitive Na⁺/H⁺ exchanger, but there may be a small role for a DIDS-sensitive Cl⁻/HCO₃⁻ exchange mechanism. These results are, in general, strikingly similar to results from neurons in CO₂-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 pHi values.** The pHi of chemosensitive and nonchemosensitive neurons was not significantly different under steady-state conditions (control saline, pHi 7.8) in any of our experiments. The resting steady state pHi varied between 7.4 and 7.5. This value is similar to pHi 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 pHi values measured within 3 min of applying each stimulus were similar among neurons from the chemoreceptor region and nonchemoreceptor regions. Therefore, any differences in pHi recovery (see below) cannot be attributed to differences in the initial intracellular or extracellular pH. However, the lack of differences in pHi among chemoreceptor and nonchemoreceptor regions during acidic stimulation is at odds with some previous work. When pHi was changed from 7.48 to 7.30 in the experiments described by Ritucci et al. (24), pHi fell by ~83% of the fall in pHe in the NTS; the reduction in pHi was ~33% of the fall in pHe in the VLM. In contrast, pHi fell by only 4–22% of the change in pHe in nonchemosensitive regions (the inferior olivary and hypoglossal nucleus). In isolated glomus cells of the rabbit carotid body, which are CO₂ sensitive, pHi fell by ~60–70% of the change in pHe during hypercapnic acidosis (5). The usual change in pHi is ~20–30% of the change in pHe in other nonchemosensitive tissues (see Ref. 5 for a complete list of references). In the snail neurons, the change in pHi was 71% of the change in pHe when pHe 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 pHi for this change in pHe was not different between chemoreceptor and nonchemoreceptor cells.

The rate of pHi 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 pHᵢ 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 H. 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 caridot 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 pHₑ and pHᵢ 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⁺/Cl⁻-dependent 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$_i$ 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$_i$-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$_a$ 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$_i$ regulatory mechanisms, the results may be consistent with greater inhibition of Na$^+$/H$^+$ exchange by pH$_i$ inhibition of Na$^+$/H$^+$ exchange that may be present in chemoreceptor regions even at normal, control pH$_i$ values. However, the results in Helix are susceptible to another interpretation. pH$_i$ 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$_i$ regulatory capacity (e.g., less expression of the Na$^+$/H$^+$ exchanger per neuron).

**Perspectives**

**pH$_i$ regulation and ventilatory control.** In our previous electrophysiological studies of CO$_2$-chemosensitive neurons, we found very few intrinsically CO$_2$-sensitive neurons in each chemosensitive area, perhaps 8–12 neurons (14). It was our expectation that poor pH$_i$ regulation might be present only in a few cells in the chemosensitive area of *H. aspersa*, and we did find that poor pH$_i$ regulation among neurons in the subesophageal ganglia was significantly ($P = 0.007$) segregated and much more likely in neurons confined to the CO$_2$-sensitive area. However, the segregation was not perfect; many neurons outside the CO$_2$-sensitive area demonstrated poor pH$_i$ 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$_i$ recovery during hypercapnia, but found poor pH$_i$ 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$_i$ 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$_i$ regulatory profiles will be an insensitive marker of chemosensitivity: poor pH$_i$ regulation is ubiquitous, electrophysiological evidence of CO$_2$ chemosensitivity is more circumscribed. We conclude that a delayed or flat pH$_i$ recovery profile during intracellular and extracellular acidification is a necessary, but not sufficient, condition for CO$_2$ chemoreceptor neurons in both molluscan and murine preparations.

If neurons with flat pH$_i$ regulatory profiles during hypercapnic acidosis play an important role in CO$_2$ chemoosensory regulation of ventilation (and that is certainly our hypothesis), then the whole animal ventilatory responses to manipulations of pH$_i$ 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 CO$_2$ (20). These results imply that DIDS reduced the pH in or about chemoreceptor cells. However, DIDS had no effect on pH$_i$ or pH$_i$ 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$_i$ or pH$_i$ 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 CO$_2$ (21). The effects of amiloride on pH$_i$ and pH$_i$ regulation in chemoreceptor area neurons in the rat brain stem are the exact opposite of those expected: amiloride did not change pH$_i$ under control, normocapnic conditions but did reduce pH$_i$ 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$_i$ responses is not irrefutable. For example, the DIDS effects in the whole animal might reflect changes in pH$_i$ regulation originating in nonchemoreceptor areas that nonetheless alter pH$_a$ 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$_i$. 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$_i$ regulation is poor, are actually CO$_2$ chemosensors, although that is our working hypothesis. Nonetheless, the lack of correlation between pharmacological manipulation of the whole animal ventilatory responses to CO$_2$ and single-neuron pH$_i$ regulation is disconcerting for any theory of respiratory control that posits a key role for pH$_i$ in chemoreceptor areas.

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