Response of membrane potential and intracellular pH to hypercapnia in neurons and astrocytes from rat retrotrapezoid nucleus

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Ritucci, Nick A., Joseph S. Erlichman, J. C. Leiter, and Robert W. Putnam. Response of membrane potential and intracellular pH to hypercapnia in neurons and astrocytes from rat retrotrapezoid nucleus. Am J Physiol Regul Integr Comp Physiol 289: R851–R861, 2005.—We compared the responses of mcRTN neurons to HA (decreased pHi and pHo) and isohydric hypercapnia (decreased pHi with constant pHo). Neurons excited by HA (firing rate increased 167% and 38% of irRTN neurons (6/16), increasing firing rate by 167 ± 75% (chemosensitivity index, CI, 256 ± 72%) and 310 ± 93% (CI 292 ± 50%), respectively. These responses did not vary throughout neonatal development. We compared the responses of mcRTN neurons to HA (decreased pHi and pHo) and isohydric hypercapnia (IH; decreased pHi with constant pHo). Neurons excited by HA (firing rate increased 156 ± 46%; n = 5) were similarly excited by IH (firing rate increased 167 ± 38%; n = 5). In astrocytes from both RTN areas, HA caused a maintained intracellular acidification of 0.17 ± 0.02 pH unit (n = 6) and a depolarization of 5 ± 1 mV (n = 12). In summary, many neurons (42%) from the RTN are highly responsive (CI 248%) to HA; this may reflect both synaptically driven and astrocyte responses among a distinct region of the retrotrapezoid nucleus (RTN). The mediocaudal RTN (mcRTN), and more intermediate and rostral RTN areas (irRTN) in medullary brain slices from neonatal rats. Hypercapnic acidosis (HA) caused pHo to decrease from 7.45 to 7.15 and a maintained intracellular acidification of 0.15 ± 0.02 pH unit in 90% of neurons from both areas (n = 16). HA excited 44% of mcRTN (7/16) and 38% of irRTN neurons (6/16), increasing firing rate by 167 ± 75% (chemosensitivity index, CI, 256 ± 72%) and 310 ± 93% (CI 292 ± 50%), respectively. These responses did not vary throughout neonatal development. We compared the responses of mcRTN neurons to HA (decreased pHi and pHo) and isohydric hypercapnia (IH; decreased pHo with constant pHi). Neurons excited by HA (firing rate increased 156 ± 46%; n = 5) were similarly excited by IH (firing rate increased 167 ± 38%; n = 5). In astrocytes from both RTN areas, HA caused a maintained intracellular acidification of 0.17 ± 0.02 pH unit (n = 6) and a depolarization of 5 ± 1 mV (n = 12). In summary, many neurons (42%) from the RTN are highly responsive (CI 248%) to HA; this may reflect both synaptically driven and intrinsic mechanisms of CO2 sensitivity. Changes of pH are thought to play such a critical role in CO2 chemosensitivity, simultaneous measurement of membrane potential (Vm) and pH of cells in chemosensitive regions provides a powerful technique to determine the mechanism(s) of chemosensitivity. Such studies have recently been done in another chemosensitive area, the locus coeruleus (16), and the neural activity of cells within the locus coeruleus was best correlated with changes in pHo. In the current study, we simultaneously measured Vm and pHo in individual neurons of the RTN to characterize their CO2 chemosensitivity. Recently, the mediocaudal area of the RTN (composed of ~300 neurons) was tentatively identified as the site of the ventral medullary chemoreceptors first identified in the early 1960s (26, 29). This suggestion is at odds with evidence that indicates the entire RTN is involved in the control of breathing (6, 21, 23–25, 31, 33, 36, 37, 55, 60). Therefore, we chose to divide the RTN into two areas to determine whether there were differences in the response to hypercapnia of neurons within each area: the mediocaudal area discussed above and more intermediate and rostral areas of the RTN. The mechanism of CO2 chemosensitivity in mediocaudal RTN neurons was recently suggested to involve inhibition of a TASK channel (29). TASK channels are inhibited by decreased pHo and we exposed mediocaudal RTN neurons to both hypercapnic acidosis (decreased pHo and constant pHi) and isohydric hypercapnia (decreased pHi and constant pHo) to
assess the possible role of TASK channels in chemosensory neurons from the RTN.

There is evidence that suggests astrocytes may be involved in CO2 chemosensitivity (14, 18, 20). The role that astrocytes play in the central nervous system is much more extensive than once thought and includes the control and regulation of many substances in the extracellular space, such as glutamate, K+, and H+ (11, 22, 48). It has been proposed that astrocytes contribute to the mechanism of CO2 chemosensitivity by acidifying the extracellular environment during hypercapnia, thereby magnifying the signal to the central chemoreceptors (14, 20). We studied this possibility by simultaneously measuring the responses of Vm and pH to hypercapnic acidosis in RTN astrocytes.

Preliminary reports of these findings have been published previously (47, 52).

MATERIALS AND METHODS

Solutions and materials. Control solution contained (in mM) 124 NaCl, 3 KCl, 1.2 Na2HPO4, 1.3 MgSO4, 2.4 CaCl2, 26 NaHCO3, and 10 glucose and was equilibrated with either 5% CO2-95% O2 (pH \~7.45 at 37°C) or 10% CO2-90% O2 (hypercapnic solution; pH \~7.15 at 37°C). For the isohydric hypercapnic solution, NaHCO3 concentration was increased to 52 mM (isosmotically replacing NaCl) and equilibrated with 10% CO2-90% O2 (pH \~7.45 at 37°C). The pH calibration solution contained (in mM) 127 KCl, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, 26 K-HEPES, 10 glucose, and 0.004 nigericin (pH 7.4 at 37°C). Nigericin was purchased from Sigma and was added from a 16.7 mM stock solution (in DMSO). Pyranine (8-hydroxy- pyrene-1, 3,6-trisulfonic acid) was purchased from Molecular Probes and was added from a 4 mM stock solution (in water). Fluo-4 was also purchased from Molecular Probes and was prepared as a 5 mM stock solution (in DMSO), which was then added to control solution at a final concentration of 5 \mu M.

Preparation of medullary brain slices. All animal procedures are in agreement with the Wright State University Institutional Animal Care and Use Committee guidelines and were approved by the committee (AAALAC no. A3632-01). Transverse brain slices (200–300 \mu m) were prepared from preweanling Sprague-Dawley rats (postnatal days P0–P17) beginning at the most caudal level of the facial nucleus and extending rostrally for \~600–900 \mu m (Fig. 1). Slices were cut into ice-cold control solution with the use of a vibratome (Pelco 101, series 1000) and subsequently stored at room temperature. Experiments began at least 1 h after slicing. Individual slices were placed in a superfusion chamber (~1.0-ml volume), which was on the stage of an upright Nikon Eclipse E600 microscope, immobilized with a grid of nylon fibers, and superfused at \~4 ml/min with control solution (37°C).

Visualization of brain slices. Individual RTN neurons and astrocytes were visualized using infrared video microscopy. A \times60 water-immersion objective (W.D. 3 mm, N.A. 0.8) was used during these experiments and equipped with Hoffman Modulated Contrast optics. Light was directed to a Nikon multi-image port module equipped with a 505-nm dichroic mirror, which allowed 100% of the infrared image and 100% of the fluorescence image (see Imaging of pyranine-loaded slices) to each port simultaneously. The infrared image was then directed to a Sony charge-coupled device (CCD) Iris video camera and displayed on a Sony video monitor.

Imaging of pyranine-loaded slices. The pH-sensitive dye pyranine was loaded into individual RTN neurons and astrocytes with whole cell pipettes (see Whole cell recordings). Pyranine-loaded cells were excited (with light from a xenon arc lamp) alternately at 450 ± 10-nm (pH sensitive) and 415 ± 10-nm (pH insensitive) wavelengths with a Sutter Lambda 10-2 filter wheel. An acquisition took about 2 s and was repeated at 20- to 60-s intervals. There was no excitation light between acquisitions. Emitted fluorescence at 515 ± 10 nm (all filters from Omega Optical) was directed to the Nikon multi-image port module and then to a GenIIISys image intensifier and a CCD100 camera (both from Dage-MTI). The subsequent fluorescence images were collected and processed using Metafluor 4.6r5 software (Universal Imaging), and the 450/415 fluorescence ratios (R450/415) were determined. At the end of each experiment, we performed a one-point calibration at pH 7.4 using a high-K+/nigericin calibration solution. This calibration allowed us to normalize the R450/415 values (N450/415) and convert N450/415 into pH, by using the following calibration equation from previous studies (28, 53):

$$\text{pH} = 7.5561 + \log \left( \frac{N450}{N415} \right)$$

$\text{Fluo-4 loading of slices.}$ The fluorescent dye flou-4 is preferentially loaded by glia (43). Therefore, slices were loaded with flou-4 to aid identification of astrocytes. Slices were incubated in control solution containing 5 \mu M of the membrane-permeant form of flou-4, flou-4 AM, for 1 h in the dark at 37°C. Slices were subsequently washed and stored in fresh control solution at room temperature until experiments began (i.e., at least a 30-min wash time). Fluo-4 fluorescence was imaged by exciting the dye at 490 nm and collecting the emitted fluorescence at 520 nm. Fluo-4 fluorescence did not interfere with pyranine fluorescence: fluo-4 fluorescence was absent during imaging with our pyranine emission cube (data not shown). In later experiments, astrocytes were visually identified without flou-4 loading.
Whole cell recordings. Whole cell pipettes (5 MΩ) were fabricated from borosilicate glass (TW150–3; World Precision Instruments) with the use of a Narishige PP-830 dual-stage pipette puller and were filled with a solution containing (in mM) 130 K-glucanone, 1 MgCl₂, 10 HEPES, 0.4 EGTA, 2 Na₂ATP, 0.3 Na₂GTP, and 0.2 pyranine (pH 7.35 at 37°C). This filling solution, which has no added Ca²⁺ and low levels of EGTA, reduces washout in chemosensitive neurons (7, 17). The pipette holder contained a Ag-AgCl wire, and the circuit was completed with a Ag-AgCl wire placed (downstream to the brain slice) in the superfusion bath. Slight positive pressure was applied to the whole cell recording pipette. To achieve a tight seal, we initially manipulated the pipette to touch the membrane of the soma. Tight seals were attempted solely on the outer edge of the soma so that the fluorescence from pyranine in the pipette would not interfere with the fluorescence of pyranine in the soma. Once the pipette touched the outer edge of the soma, negative pressure was applied to the pipette to form a tight (gigaohm) seal with the cell membrane. The tight seal was then ruptured to achieve the whole cell configuration. Pyranine diffused from the whole cell pipette into the cell, and stable Rₚ values were achieved within ~15 min (28).

Perforated-patched recordings. Pipettes (5 MΩ) were fabricated from borosilicate glass (TW150–3; World Precision Instruments) with the use of a Narishige PP-830 dual-stage pipette puller and contained (in mM) 130 K-methanesulfonate, 20 KCl, 5 HEPES, and 1 EGTA as well as 240 μg/ml amphotericin B (pH ~7.35 at 37°C). The pipette was backfilled with this solution, while the tip of the pipette was filled with an amphotericin B-free solution. Amphotericin B was purchased from Sigma and was added from a 60 mg/ml stock solution (in DMSO).

Electrophysiological recordings were conducted in current-clamp mode, and Vₘ was measured using a Dagan BVC-700 amplifier. As such, Vₘ represents a time-averaged value of all potentials and was averaged over at least a 1-min period with pCLAMP software. A healthy neuron had a resting Vₘ of between ~30 and ~60 mV. All neurons were spontaneously active. A healthy astrocyte had a resting Vₘ of between ~70 and ~80 mV, and action potentials could not be evoked upon injection of depolarizing currents.

Data analysis. Vₘ data were saved to both a digital VCR (model 400; Vetter) and Axoscope software (version 8.0) for later analysis. Firing rates were obtained (10-s bins) using a window discriminator/integrator (Winston Electronics) and were determined by averaging firing rate values over at least a 1-min period just before switching to a new solution. We expressed the magnitude of chemosensitivity by calculating the chemosensitivity index (CI) as described by Wang et al. (64). CI was calculated for the response of each neuron and then averaged across all neurons. The original calculation of CI is based on a change of pHo of 0.2 pH unit (64). Because we use hypercapnic acidosis conditions that result in a change of pHo of 0.3 pH unit, we adjusted our calculated CI based on a change of 0.2 pH unit so that it would be directly comparable to CI values calculated for chemosensitive neurons from other brain stem regions (46, 64). A value for CI cannot be calculated when using isohydric hypercapnia (because there is no change in pHl). Therefore, we also expressed the percentage by which firing rate increased in each condition according to the equation 

\[ \% \text{FR increase} = \left( \frac{\text{FR}_{\text{hypercapnia}} - \text{FR}_{\text{control}}}{\text{FR}_{\text{control}}} \right) \times 100 \]

where FR is firing rate. All values are given as means ± SE. Significant differences between two means were determined using Student’s paired t-tests (significance at P < 0.05), and the distribution of percent chemosensitive neurons was compared with a χ² test.

RESULTS

Whole cell recordings of RTN neurons during hypercapnic acidosis. All recordings of RTN neurons and astrocytes were performed on cells that were no deeper than 200 μm from the ventral surface and were typically one focal plane below the slice surface. We studied two populations of RTN neurons (Fig. 1, modified from Ref. 41). One small population (~300 cells) lies in the most caudal slice through the RTN (i.e., bregma 11.7–11.4) and is directly ventral and at the medial aspect of the facial nucleus (29). This area is well vascularized, and the presence of vessels aided in its identification. We refer to this area as the mediocaudal RTN (mcRTN). The other population of neurons lies in more intermediate and rostral slices (i.e., bregma 11.4–10.5) and therefore is referred to as the irRTN.

Firing rate, Vₘ, and pHᵢ were measured in neurons from the mcRTN in the whole cell configuration (Fig. 2). Slices were initially superfused with control solution until stable values of firing rate, Vₘ, and pHᵢ were achieved. Upon superfusion with hypercapnic acidosis solution, three of eight neurons (38%) increased their firing rate from an initial value of 0.90 ± 0.38 Hz (range 0.2 to 1.5 Hz) to 2.8 ± 0.44 Hz, which corresponds to a CI of 314 ± 147%. These three neurons also depolarized ~6 mV, from an initial value of ~45.0 ± 1.7 mV (range ~42 to ~48 mV) to ~38.8 ± 0.9 mV. In addition, there was a maintained intracellular acidification of 0.12 pH unit, from an initial value of 7.31 ± 0.01 to 7.19 ± 0.02. Upon return to control solution, firing rate (0.73 ± 0.27 Hz), Vₘ (~46 ± 2.1 mV), and pHᵢ (7.30 ± 0.02) returned toward their initial values. In the other five neurons tested (Fig. 3), there was once
again a maintained intracellular acidification during hypercapnic acidosis, from an initial value of 7.32 ± 0.01 to 7.20 ± 0.01. However, these neurons did not exhibit a change in firing rate [from 1.54 ± 0.41 Hz (range 0.2 to 3.0 Hz) to 1.54 ± 0.41 Hz] or $V_m$ [from −43.6 ± 2.0 mV (range −38 to −50 mV) to −42.4 ± 2.2 mV]. Upon return to control solution, pH, returned to its initial value (7.31 ± 0.02). Firing rate (1.54 ± 0.41 Hz) and $V_m$ (−43.4 ± 1.4 mV) remained unchanged.

Firing rate, $V_m$, and pH, also were measured in neurons from the irRTN with whole cell pipettes. In two of eight neurons (25%), there was an increase in firing rate upon exposure to hypercapnic acidosis from an average initial value of 0.65 Hz (range 0.3 to 1.0 Hz) to 4.0 Hz, which corresponds to a CI of 413 ± 149%. In addition, these neurons depolarized ~3 mV, from an average initial value of −46.6 mV (range −37 to −52 mV) to −43.5 mV. There was also a maintained intracellular acidification of 0.17 pH unit, from an average initial value of 7.29 to 7.12. Upon return to control solution, average firing rate (1.0 Hz), $V_m$ (~42.0 mV), and pH, (7.26) returned toward their initial values. In the other six neurons tested, firing rate [from 1.45 ± 0.73 Hz (range 0.2 to 5.0 Hz) to 1.45 ± 0.73 Hz] and $V_m$ [from −44.8 ± 1.8 mV (~41 to −52 mV) to −45.7 ± 3.2 mV] remained unchanged. However, there was once again a maintained intracellular acidification during hypercapnic acidosis from an initial value of 7.34 ± 0.03 to 7.20 ± 0.09. Upon return to control solution, pH, returned to its initial value (7.29 ± 0.07) and firing rate (1.37 ± 0.65 Hz) and $V_m$ (−42.5 ± 4.3 mV) again remained unchanged.

**Perforated-patch recordings of RTN neurons during hypercapnic acidosis.** Because whole cell pipettes can cause washout of soluble intracellular components, which could be important in the signaling mechanism of CO2 chemosensitivity (10, 17, 49), we repeated the experiments using perforated-patch recordings, avoiding issues of washout of the $V_m$ response to hypercapnia. Firing rate and $V_m$ were measured in neurons from the mcRTN, using perforated-patch pipettes. Slices were initially superfused with control solution until stable values of firing rate and $V_m$ were achieved. Upon superfusion with hypercapnic acidosis solution, four of eight neurons (50%) increased their firing rate from an initial value of 2.0 ± 0.87 Hz (range 0.3 to 4.0 Hz) to 4.7 ± 1.5 Hz, which corresponds to a CI of 210 ± 36%. These neurons also depolarized ~5 mV, from an initial value of −42.7 ± 6.3 mV (range −34 to −55 mV) to −37.3 ± 5.8 mV. Upon return to control solution, firing rate (2.3 ± 0.73 Hz) and $V_m$ (~39.3 ± 6.0 mV) returned toward their initial values. In the other four neurons tested, there was no change in firing rate [from 2.40 ± 0.47 Hz (range 1.0 to 3.0 Hz) to 2.38 ± 0.47 Hz] or $V_m$ [from −34.5 ± 5.4 mV (range −33 to −46 mV) to −38.5 ± 4.3 mV] during exposure to hypercapnic acidosis. Upon return to control solution, firing rate (2.0 ± 0.41 Hz) and $V_m$ (~36.8 ± 5.7 mV) remained unchanged.

Firing rate and $V_m$ also were measured in neurons from the irRTN, using perforated-patch pipettes. In four of eight neurons (50%), there was an increase in firing rate upon exposure to hypercapnic acidosis, from an initial value of 1.2 ± 0.91 Hz (range 0.2 to 4.0 Hz) to 3.7 ± 2.7 Hz, which corresponds to a CI of 241 ± 26%. These neurons depolarized ~4 mV, from an initial value of −39.7 ± 4.5 mV (range −38 to −46 mV) to −36.0 ± 5.5 mV. Firing rate (0.9 ± 0.6 Hz) and $V_m$ (~38.0 ± 7.4 mV) returned toward their initial values under control conditions. In the other four neurons tested, firing rate [from 1.5 ± 0.35 Hz (range 0.5 to 3.0 Hz) to 1.3 ± 0.35 Hz] and $V_m$ [from −42.3 ± 4.8 mV (range −32 to −55 mV) to −44.3 ± 4.0 mV] remained unchanged during hypercapnic acidosis exposure and upon return to control solution (firing rate of 1.3 ± 0.35 Hz and $V_m$ of −40.0 ± 5.0 mV).

**Age effects on chemosensitivity of RTN neurons.** There could be concerns that our findings are confounded by developmental changes in RTN neuronal chemosensitivity in the neonatal rats used in this study. Thus we examined the CO2 sensitivity of all excited neurons (regardless of method of study and location within the RTN) from our neonatal rats (P0–P17). All neurons studied were divided into groups from rats either younger than P10 or older than P10. The percentage of neurons activated by hypercapnia from rats younger than P10 was 40% (10 activated of 25 studied), and it was 42% (8 activated of 19 studied) in neurons from rats older than P10. These values are not significantly different ($P > 0.12$). The remainder of the neurons did not respond to hypercapnia with a change in firing rate. We did not observe any RTN neurons whose firing rate was inhibitable by hypercapnia. We also calculated the CI of the neurons from the different age groups to determine their relative chemosensitivity. The CI of neurons activated by CO2 was 209 ± 13%
in animals younger than P10 and 296 ± 65% in animals older than P10. These values also are not significantly different (P > 0.1). Thus chemosensitive neurons in the RTN do not appear to show developmental changes in their chemosensitivity during postnatal development.

Whole cell recordings of RTN neurons during isohydric hypercapnia. Because pHo has been suggested to play a role in the response of RTN neurons to hypercapnia (29), we exposed the slices to both hypercapnic acidosis (which results in a decrease in both pHi and pHo) and isohydric hypercapnia (which results in a decrease in pHi with a constant pHo) while measuring Vm and pHi using whole cell pipettes (Fig. 4). These experiments were performed solely in the mcRTN. Slices were initially superfused with control solution until stable values of firing rate, Vm, and pHi were achieved. Upon superfusion with hypercapnic acidosis solution, 5 of 11 neurons (45%) increased their firing rate by 156 ± 46% from an initial value of 1.5 ± 0.35 Hz (range 0.5 to 2.5 Hz) to 3.4 ± 0.68 Hz (Fig. 4). These neurons also depolarized ~3 mV, from an initial value of -43.5 ± 5.2 mV (range -39 to -48 mV) to -40.4 ± 4.5 mV. In addition, there was a maintained intracellular acidification of 0.15 pH unit, from 7.31 ± 0.01 to 7.16 ± 0.02 (Fig. 4). Upon return to control solution, firing rate (1.9 ± 0.43 Hz), Vm (-44.0 ± 3.0 mV), and pHi (7.30 ± 0.02) returned to their initial values. Slices were then superfused with isohydric hypercapnic solution. The five neurons that were excited by hypercapnic acidosis also were excited by isohydric hypercapnia (Fig. 4). Firing rate increased by 167 ± 38% from 1.9 ± 0.43 Hz to 4.8 ± 1.09 Hz (Fig. 4). These neurons also depolarized ~3 mV, from -44.0 ± 3.0 mV to -41.2 ± 3.5 mV. The pHi response consisted of an initial decrease of 0.10 ± 0.02 pH unit, from an original value of 7.30 ± 0.01 to 7.20 ± 0.03, that was followed by pHi recovery (Fig. 4). Upon return to control solution, firing rate (1.9 ± 0.57 Hz) and Vm (-44.5 ± 3.5 mV) returned to their initial values, whereas pHi overshot its initial value to a value of 7.35 ± 0.02, which is consistent with pHi recovery. A comparison of these five neurons shows that their firing rate response to hypercapnic acidosis (156 ± 46%) did not differ significantly (P > 0.85) from their firing rate response to isohydric hypercapnia (167 ± 38%).

In the remaining six neurons tested (Fig. 5), there was once again a maintained intracellular acidification of 0.15 pH unit during hypercapnic acidosis, from an initial value of 7.29 ± 0.01 to 7.14 ± 0.02. However, these neurons did not exhibit a change in firing rate [from 1.3 ± 0.55 Hz (range 0.2 to 3.5 Hz) to 1.3 ± 0.85 Hz] or Vm [from -42.3 ± 4.8 mV (range -36 to -48 mV) to -42.0 ± 5.1 mV] during hypercapnic acidosis exposure. Upon return to control solution, pHi returned toward its initial value (7.28 ± 0.02). Firing rate (1.5 ± 0.62 Hz) and Vm (-41.8 ± 4.9 mV) remained unchanged. During exposure to isohydric hypercapnia, the pHi response consisted of a decrease of 0.10 ± 0.03 pH unit, from an initial value of 7.28 ± 0.01 to 7.18 ± 0.03, that was followed by pHi recovery (Fig. 5). These neurons, again, did not exhibit a change in firing rate (from 1.5 ± 0.62 Hz to 1.6 ± 0.55 Hz) or Vm (from -41.8 ± 4.9 mV to -42.0 ± 5.1 mV) during exposure to isohydric hypercapnia. Upon return to control solution, firing rate (1.4 ± 0.72 Hz) and Vm (-42.1 ± 4.9 mV) remained unchanged, whereas pHi overshot its initial value to a value of 7.36 ± 0.02 (Fig. 5), which is consistent with pHi recovery.

Chemosensitivity in RTN neurons. To characterize the chemosensitivity of RTN neurons, we pooled our data from various regions, techniques, and ages and determined the percentage of neurons that responded to hypercapnia with an increased firing rate (no neuron was found to be inhibited by hypercapnia) and the magnitude of their response (as determined by the CI). In all cases, the percentage of neurons that responded to hypercapnic acidosis did not differ significantly with region, measuring technique, or age. Thus, pooling all data, we found that 18 of a total 43 RTN neurons, or 42%, responded to hypercapnic acidosis with an increased firing rate (>20%). Furthermore, the magnitude of the response (i.e., the CI) of the 18 chemosensitive RTN neurons was 248 ± 30%. These values are then the best estimates for the percentage of RTN neurons that are chemosensitive and the magnitude of their response to hypercapnia.

Fluo-4 loading. To aid in the identification of astrocytes, we loaded slices with the fluorescent dye fluo-4 (Fig. 6). Loaded cells were either spherical or irregularly shaped and were ~10 μm in size. The loaded cells of interest were visualized no deeper than 200 μm from the ventral surface (Fig. 6) and were
typically one focal plane below the slice surface. Once a fluo-4-loaded cell was identified and so assumed to be an astrocyte, electrophysiological recordings were performed (see below). Every fluo-4-loaded cell met our electrophysiological criteria for astrocytes (see Whole cell recordings of RTN astrocytes during hypercapnic acidosis). Once we became proficient at identifying astrocytes, fluo-4 loading was no longer necessary.

Whole cell recordings of RTN astrocytes during hypercapnic acidosis. We also wanted to see what the effect of hypercapnic acidosis was on the \( V_m \) and \( pHi \) of astrocytes from both the mcRTN and irRTN. Our criteria for confirming that we were patched on an astrocyte were silent cells with a highly negative \( V_m \), the inability to evoke an action potential with depolarizing current injection, and the appearance of depolarization-induced alkalinization (Fig. 7). All cells that we patched that lacked the ability to generate action potentials also had a highly negative \( V_m \) and exhibited depolarization-induced alkalinization. Because the changes seen in \( V_m \) and \( pHi \) during exposure to hypercapnic acidosis were the same in both areas during the use of whole cell pipettes, the data (\( n = 6 \)) from both areas were pooled. Astrocytes, like neurons, acidified by 0.15 pH unit (from 7.32 ± 0.02 to 7.17 ± 0.02) and maintained that acidification for the entire duration of the exposure to hypercapnic acidosis (Fig. 7). The membrane potential of astrocytes, which was far more hyperpolarized than the resting \( V_m \) of neurons, also depolarized in response to hypercapnic acidosis by \( \approx 4 \) mV, from an initial value of \(-75.0 \pm 1.4 \) mV to \(-70.6 \pm 1.8 \) mV. Upon return to control solution, \( V_m \) returned toward its initial value (7.31 ± 0.06), as did \( pHi \) (7.31 ± 0.06) (Fig. 7). At the end of each of these experiments, we wanted to further confirm that these recordings were from astrocytes, so we depolarized the membrane with approximately +40-mV direct current and observed the change in \( pHi \). It has been shown in a previous study that depolarizing astrocytes will cause the inwardly driven electrogenic \( Na^+\text{-HCO}_3^- \) cotransporter to be activated and thus produce an intracellular alkalinization (11), termed depolarization-induced alkaliniza-

Fig. 5. Hypercapnic acidosis- and isohydric hypercapnia-insensitive mcRTN neuron. Top: integrated firing rate trace of an individual mcRTN neuron. Average integrated firing rate was 1.8 Hz before exposure and decreased (0.5 Hz) upon exposure to hypercapnic acidosis (10% \( CO_2 \), 26 mM \( HCO_3^- \), \( pH_4 \), 7.15). However, this effect was not reversible, with firing rate remaining at 0.6 Hz upon return to control solution (5% \( CO_2 \), \( pH_4 \), 7.45). Exposure to isohydric hypercapnia (10% \( CO_2 \), 52 mM \( HCO_3^- \), \( pH_4 \), 7.45) did not change firing rate (0.8 Hz), nor did return to control solution (0.9 Hz). Bottom: \( pH_i \) trace of the same neuron recorded at top. Hypercapnic acidosis resulted in a maintained intracellular acidification that returned toward its initial value upon return to control solution (5% \( CO_2 \), 26 mM \( HCO_3^- \), \( pH_4 \), 7.45). Isohydric hypercapnia resulted in an initial intracellular acidification followed by \( pH_i \) recovery. Upon return to control solution, \( pH_i \) overshot its initial value, further indicating \( pH_i \) recovery during isohydric hypercapnia. \( pH_i \) then returned toward its initial value.

Fig. 6. Fluo-4 fluorescence and Hoffman contrast images of irRTN. Left: fluo-4 fluorescence image of the irRTN. Fluorescence image shows a slice loaded with the fluorescent dye fluo-4, which preferentially loads glia. Arrow indicates a fluo-4-loaded cell. Arrowhead indicates the ventrolateral surface. Right: Hoffman differential interference contrast (DIC) image of the same area shown at left. Arrow indicates the same cell indicated by arrow at left. This cell was patched, and the membrane potential (\( V_m \)) and \( pH_i \) traces are shown in Fig. 7. Arrowhead indicates the ventrolateral surface. Note how close to the ventral surface the cells of the RTN are located.
tion (39). This is what was seen in RTN astrocytes (Fig. 7). If these cells were neurons, the depolarization would have produced an intracellular acidification (61).

Perforated-patch recordings of RTN astrocytes during hypercapnic acidosis. As previously stated, because whole cell pipettes can cause washout of the neuronal electrical response to hypercapnia, we were concerned that the use of whole cell pipettes could also alter the response of astrocytes to hypercapnia. Therefore, we repeated the whole cell pipette experiments using perforated-patch recordings. Once again, the results in astrocytes from both RTN areas were combined (n = 6), because the changes seen in V_m during hypercapnic acidosis were the same. The depolarization measured with perforated-patch pipettes was similar to that observed using whole cell pipettes. Hypercapnic acidosis resulted in a membrane depolarization of ~5 mV, from an initial value of ~77.8 ± 1.0 mV to ~72.8 ± 0.7 mV. Upon return to control conditions, V_m returned toward its initial value (~77.5 ± 1.2 mV).

DISCUSSION

There were four main findings in this study. First, a similar percentage (i.e., ~42%) of neurons was excited by hypercapnic acidosis in the mcRTN and the irRTN. Furthermore, RTN neurons from both areas, just like in other chemosensitive regions of neonates (51), had a maintained intracellular acidification during hypercapnic acidosis. Second, pH_e appears to play a major role, and decreased pH_e, a lesser role, in the response of RTN neurons to hypercapnia. Every neuron from the mcRTN that was excited by hypercapnic acidosis was also excited by isohydric hypercapnia, and every neuron that was insensitive to hypercapnic acidosis was also insensitive to isohydric hypercapnia. Third, the firing rate increase was large (CI ~250%) and was the same in response to hypercapnic acidosis and isohydric hypercapnia in both the mcRTN and irRTN. Finally, the response of astrocytes to hypercapnia was shown to be similar to that of neurons: a modest depolarization (~5 mV) along with a maintained intracellular acidification. These data indicate that the entire RTN is equally and highly chemosensitive and that astrocytes do not appear to be regulating pH_e under conditions of hypercapnic acidosis.

Distribution of CO_2-sensitive neurons within the RTN. The present study investigated the V_m and pH_e responses to hypercapnia of individual neurons and astrocytes of the mcRTN and irRTN in brain slices from neonatal rats. There were no consistent or significant differences in resting V_m or initial firing rate based on the method of study or location within the RTN. We found that 38% (3 of 8) of neurons from the mcRTN were excited by hypercapnic acidosis when whole cell pipettes were used and 50% (4 of 8) were excited by hypercapnic acidosis when perforated-patch pipettes were used. These values are somewhat higher than the values reported by Okada et al. (36), who found that 29% of RTN neurons in the same area were excited by hypercapnic acidosis when recorded with perforated-patch pipettes. Mulkey et al. (29) also performed recordings in the mcRTN. Using loose-patch recordings in a HEPES-buffered solution, they found that 46% of neurons tested were excited by decreasing pH_e to 6.9 (control pH 7.3). A few of these neurons also were excited by hypercapnic acidosis (in a CO_2/HCO_3^--buffered solution), prompting the speculation that the area from which they recorded (corresponding to the mcRTN recorded in this study) might be area M of the ventrolateral medulla (VLM) (see Ref. 26), which was first described in the early 1960s.

We also wanted to compare the CO_2 chemosensitivity of mcRTN to that of IRRTN neurons, to determine whether the mcRTN represents a specialized chemosensitive area. We found that a similar percentage of mcRTN and irRTN neurons were excited by hypercapnic acidosis (~44% vs. ~38%, respectively) and to the same extent (CI of 256 ± 72% and 310 ± 65%, respectively). These findings suggest that the
mcRTN is not particularly distinct from the rest of the RTN. This is not entirely surprising, given the fact that Mitchell’s rostral chemosensitive region of the rostral VLM (where the RTN is located) encompasses a fairly large area (27). The RTN, in particular, has been shown to be CO2 and acid chemosensitive, starting at its most caudal end and extending to its most rostral pole (6, 21, 23–25, 31, 33, 37). In addition, increased c-fos in response to hypercapnia has been seen in the caudal to rostral extent of the RTN without any particular concentration of CO2-responsive cells in the mcRTN (36, 55, 60), suggesting that much of the RTN is involved in the control of breathing.

One possible limitation of this study is that the brain slices were taken from young animals (P0–P17). Investigators study neonatal brain slices because the astrocytic proliferation, which starts about P8–P12, interferes with the access of both patch and sharp electrodes to the neurons and because the growth of the neuropil in older animals makes imaging individual neurons within the slice difficult. However, the ventilatory response to CO2 in neonates changes dramatically in this age range (45, 58, 67), and the question arises, therefore, whether these neuronal responses from neonates are representative of those from adults. We believe that they are. Our analysis of relative CO2 sensitivity in the RTN in the current study indicates that there are no differences in chemosensitivity between neurons from rats younger than P10 and those older than P10. Furthermore, c-fos studies indicate that the number of CO2-responsive neurons remains constant throughout early development in the RTN (3, 67). Thus it does not appear that there are maturational changes in the CO2 sensitivity of RTN neurons during early neonatal development.

There is one final limitation. We did not study the effect of synaptoc blockade on CO2-induced neuronal activity. Some of the neurons we studied are likely to be intrinsically CO2 sensitive, as seen with chemosensitive neurons from other brain stem areas (8, 38, 49), but some of the activity that we recorded may be synaptically driven.

**pHi, as a chemosensory stimulus.** A decrease of pHi is thought to play a major signaling role in CO2 chemosensitivity (4, 15, 16, 63). Therefore, in this study, pHi and Vm were measured simultaneously. We also wanted to see if there was a difference in pHi changes during hypercapnia. We found that hypercapnic acidosis caused a maintained intracellular acidification in ~90% of the neurons in both RTN areas, which is similar to the findings of Nottingham et al. (35), although no Vm measurements were made in that study. In our study, although nearly all RTN neurons had a maintained acidification in response to hypercapnic acidosis, only about one-half of the neurons were found to be excited by hypercapnic acidosis. Thus a maintained intracellular acidification cannot be used to define a chemosensitive neuron (see also Ref. 4), although all chemosensitive neurons do exhibit a maintained acidification during hypercapnic acidosis (46).

The above findings indicate that a chemosensitive neuron needs to be defined as one in which Vm responds (either excited or inhibited) to changes in CO2 and/or pH. This likely occurs by a change in ion channel activity. The most widely suggested chemosensitive mechanism involves inhibition of K+ channels by a decrease in pHi and/or pH during hypercapnia (9, 17, 29, 44, 65, 66, 68), which would cause an increase in excitability. The K+ channels that appear to be most sensitive to changes in pHi are the TASK channels, and the most likely candidate would be TASK-1 with a (physiological) pK of ~7.4 (12). Mulkey et al. (29) speculated that TASK channels may be involved in the chemosensitive response of mcRTN neurons. However, we found that the firing rate response of mcRTN neurons was the same upon exposure to hypercapnic acidosis (both pHi and pH decreased) and isohydric hypercapnia (pHi decreased but pH constant). This finding indicates that a decrease of pHi is sufficient to activate chemosensitive neurons in RTN and argues against inhibition of a K+ channel (i.e., TASK-1) by a decrease in pHi as an essential element in the mechanism of CO2 chemosensitivity in these neurons. Furthermore, TASK-1 expression appears to be absent in the RTN (see Fig. 2B of Ref. 2). Thus the chemosensitive response of RTN neurons is most likely mediated by other K+ channels, perhaps including those that are sensitive to changes of pHi.

As noted above, the firing rate response of RTN neurons to hypercapnic acidosis and isohydric hypercapnia was the same (Fig. 4). However, the decrease in pH during isohydric hypercapnia was less than what it was during hypercapnic acidosis, and it was not maintained (Fig. 4). This indicates that some signal(s) other than decreased pHi is likely involved in the chemosensitive response of RTN neurons. One possible additional signal is increased intracellular HCO3− concentration. Under our isohydric hypercapnic conditions, in addition to elevated CO2, there is a doubling of extracellular HCO3− concentration compared with control, which should result in elevated intracellular HCO3−. In another chemosensitive cell type, the glomus cell of the carotid body, elevated intracellular HCO3− has been shown to be involved in hypercapnia-induced excitation (59). This issue could be addressed by studying the neuronal response to metabolic acidosis (4, 16). Another possible target is the activation of L-type Ca2+ channels, which has been suggested to contribute to CO2 chemosensitivity in locus coeruleus neurons (16). The involvement of multiple signaling pathways and targets in chemosensitive neurons in general has recently been proposed (15, 46).

The study of the mechanism of CO2 chemosensitivity has focused mainly on neurons, whereas the role that astrocytes might play in CO2 chemosensitivity has largely been ignored. There is evidence that astrocytes are involved in CO2 chemosensitivity (14, 18, 20). For example, perfusion of fluorocitrate (a glial toxin) into the RTN of awake rats caused an increase in the ventilatory response to CO2 (20). We investigated the role of astrocytes in chemosensitivity by measuring the effects of hypercapnia on Vm and pHi in astrocytes from both the mcRTN and irRTN. During hypercapnic acidosis, astrocytes behaved in the same manner as neurons: a modest depolarization (~5 mV) with a maintained intracellular acidification. Thus astrocytes, like neurons, also do not exhibit pHi recovery from acidification during hypercapnic acidosis, despite the presence of numerous acid-extruding transporters in astrocytes (56). One proposed mechanism whereby astrocytes might modify chemosensitive responses of neurons is for pHi recovery in astrocytes in response to hypercapnia to amplify the acidification of pHi, increasing the firing rate of chemosensitive neurons (13). Because RTN astrocytic pHi does not recover from hypercapnic acidification (Fig. 7), such a mechanism does not seem to be involved in the chemosensitive response of RTN neurons in the neonatal period.
Just as the RTN shows heterogeneity in neuronal properties (e.g., some cells respond to hypercapnia while others do not), there is evidence for considerable heterogeneity in astrocytes. Distinct astrocytic populations have been identified in the hippocampus on the basis of differences in expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (Glu-R) and glutamate transporters (Glu-T) (62). Three populations of astrocytes can be distinguished on the basis of the presence of different K⁺ currents in caudal medullary slices from neonatal mice (19). Fukuda et al. (18) identified three populations of astrocytes on the basis of electrophysiological measurements in ventral medullary slices from older rats during isocapnic acidosis. In 44% of astrocytes, \( V_m \) slowly depolarized by \(-20\) mV. In 47% of the cells, \( V_m \) was unchanged, and in 5% of the cells, \( V_m \) slowly hyperpolarized in response to decreased pH. Finally, we identified two distinct populations of astrocytes in brain stem slices from neonatal mice: one that regulates pH in response to hypercapnic acidosis and another that does not exhibit pH regulation (Erlichman JS, unpublished observation).

In the current study, we did not see evidence for heterogeneity among astrocytes. The difference between our findings and the studies discussed above may be due to the fact that we used young rats (<20) and that, in these neonatal animals, the astrocytes may not have fully differentiated into distinct populations. The degree of depolarization-induced alkalinization that we observed (Fig. 7) is about an order of magnitude smaller than values previously observed in astrocytes (39). Depolarization-induced alkalinization is largely due to activation of electrogenic Na⁺-HCO₃⁻ cotransport (NBC) (54). Therefore, the RTN astrocytes that we studied seem to have low NBC activity, which is consistent with the lack of pH recovery in response to hypercapnic acidosis (Fig. 7). It may be that the expression of NBC increases during development, and pH recovery might occur in older astrocytes during hypercapnic acidosis, or it may be that even in younger animals there is considerable heterogeneity among astrocytes, but for whatever reason, only one type of astrocytes is readily patched.

**Perspectives**

Our findings indicate that the entire RTN contains CO₂-excitied neurons and therefore may be involved in the control of breathing, which is consistent with previous work (6, 21, 23–25, 31, 33, 36, 37, 55, 60). Our findings are somewhat at odds with the recent suggestion that there is a specialized region of the caudal RTN that is especially associated with ventilatory control (26, 29). We did note that this caudal region appears to be highly vascularized, more so than other areas of the RTN. It may be that some chemosensitive neurons within the RTN are located near blood vessels, as observed in other chemosensitive areas (5), whereas others are more distant. If this vascular heterogeneity exists, it could contribute to functional differences in sensitivity among neurons that actually share the same intrinsic CO₂ sensitivity. This would make the ventilatory response to CO₂ some weighted average of the Paco₂ in multiple locations in the brain. Therefore, chemoreceptors associated with vessels would respond to rapid changes in CO₂, and chemoreceptors not associated with vessels would respond to a more stable and slower changing tissue CO₂ level. Although this idea is attractive, there appears to be extensive vascularization of the entire RTN with numerous penetrating arteries branching from the basilar artery (40). The relationship between chemosensitive neurons and blood vessels in the RTN warrants further study.

Our results offer insights into the cellular chemosensitive signaling mechanisms as well. It appears that extracellular acidification plays at most a minor role in chemosensitive signaling, because RTN neurons were equally excited by hypercapnic acidosis and isohydric hypercapnia. This occurred despite the fact that hypercapnic acidosis induced a larger and more maintained intracellular acidification than isohydric hypercapnia. These findings clearly imply that although intracellular acidification most likely plays a significant role in the chemosensory response, it is not the sole signal. It is not clear what other intracellular signals are involved in chemosensitive RTN neurons, but the idea that chemosensitivity involves multiple factors has recently been proposed (17, 46).

We have made the first simultaneous measurements of \( V_m \) and pH in astrocytes from chemosensitive brain stem regions. We found no evidence for pH recovery in response to hypercapnic acidosis-induced acidification. If astrocytes had shown pH recovery, and thus caused a larger extracellular acidification, it would have offered a mechanism by which astrocytes could modulate chemosensitivity. However, hypercapnic acidosis may alter astrocyte function in other ways besides intracellular acidification, and this could be the basis for modulation of chemosensitivity by astrocytes. It is also possible that astrocytes are heterogeneous in the RTN and that we have studied only one subtype. Finally, we have only studied RTN neurons from neonatal animals. Considerable developmental changes could occur that might result in dramatic changes in chemosensitivity (45). Thus studies of RTN neurons from adult animals should prove interesting.

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**REFERENCES**

R860 RESPONS  TO  HYPERCAPNIA  IN  RTN  NEURONS  AND  ASTROCYTES


