J Physiol 596.17 (2018) pp 4033–4042

The Journal of Physiology

Bicarbonate directly modulates activity of chemosensitive neurons in the retrotrapezoid nucleus

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Edited by Harold Schultz and Benedito Machado

Key Points

- Changes in CO_2 result in corresponding changes in both H^+ and HCO_3^- and despite evidence that HCO_3^- can function as an independent signalling molecule, there is little evidence suggesting HCO_3^- contributes to respiratory chemoreception.
- We show that HCO_3^- directly activates chemosensitive retrotrapezoid nucleus (RTN) neurons.
- Identifying all relevant signalling molecules is essential for understanding how chemoreceptors function, and because HCO_3^- and H^+ are buffered by separate cellular mechanisms, having the ability to sense both modalities adds additional information regarding changes in CO_2 that are not necessarily reflected by pH alone.
- HCO_3^- may be particularly important for regulating activity of RTN chemoreceptors during sustained intracellular acidifications when TASK-2 channels, which appear to be the sole intracellular pH sensor, are minimally active.

Abstract Central chemoreception is the mechanism by which the brain regulates breathing in response to changes in tissue CO_2/H^+. The retrotrapezoid nucleus (RTN) is an important site of respiratory chemoreception. Mechanisms underlying RTN chemoreception involve H^+-mediated activation of chemosensitive neurons and CO_2/H^+-evoked ATP-purinergic signalling by local astrocytes, which activates chemosensitive neurons directly and indirectly by maintaining vascular tone when CO_2/H^+ levels are high. Although changes in CO_2 result in corresponding changes in both H^+ and HCO_3^- and despite evidence that HCO_3^- can function as an independent signalling molecule, there is little evidence suggesting HCO_3^- contributes to respiratory chemoreception. Therefore, the goal of this study was to determine whether HCO_3^- regulates activity of chemosensitive RTN neurons independent of pH. Cell-attached recordings were used to monitor activity of chemosensitive RTN neurons in brainstem slices (300 μm thick) isolated from rat pups (postnatal days 7–11) during exposure to low or high concentrations of HCO_3^-. In a subset of experiments, we also included 2′,7′-bis(2carboxyethyl)-5-(and 6)-carboxyfluorescein

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DOI: 10.1113/JP276104

(BCECF) in the internal solution to measure pHi under each experimental condition. We found that HCO_3^- activates chemosensitive RTN neurons by mechanisms independent of intracellular or extracellular pH, glutamate, GABA, glycine or purinergic signalling, soluble adenylyl cyclase activity, nitric oxide or KCNQ channels. These results establish HCO_3^- as a novel independent modulator of chemoreceptor activity, and because the levels of HCO_3^- along with H^+ are buffered by independent cellular mechanisms, these results suggest HCO_3^- chemoreception adds additional information regarding changes in CO_2 that are not necessarily reflected by pH.

(Resubmitted 30 April 2018; accepted after revision 14 May 2018; first published online 4 June 2018)
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Introduction

Breathing is maintained unconsciously by the ability of discrete subsets of cells (neurons and astrocytes) to sense the accumulation of tissue CO_2 (i.e. respiratory chemoreceptors) as would occur during hypoventilation or apnoeic events and relay this information to downstream components of the respiratory circuit to increase rate and depth of breathing (Nattie & Li, 2012). A brainstem region called the retrotrapezoid nucleus (RTN) is important for multiple aspects of breathing including respiratory chemoreception (Guyenet *et al.* 2012; Guyenet & Bayliss, 2015). RTN neurons are thought to primarily respond to CO_2 via the proxy of H^+ by inhibition of TASK-2 potassium channels (Wang *et al.* 2013) and activation of GPR4 (Kumar *et al.* 2015). However, dissociated chemosensitive RTN neurons show differential firing responses to pH in the absence and presence of CO_2 /HCO_3^-; RTN chemoreceptors show a pH sensitivity of ∼5 Hz/pH in HCO_3^--free Hepes buffer, whereas in HCO_3^- solution their sensitivity increased to ∼8 Hz/pH (Wang *et al.* 2013). Furthermore, in rat brain slices chemosensitive RTN neurons showed similar firing responses to 10% CO_2 under control conditions (HCO_3^- = 26 mM) and when the concentration of HCO_3^- was increased (HCO_3^- = 52 mM) to maintain extracellular pH (pHo) and dampen changes in pHi (isohydric hypercapnia) (Ritucci *et al.* 2005). Together, these results suggest pH *per se* is not the sole transducer for CO_2 detection by RTN neurons. Given that changes in CO_2 result in corresponding changes in H^+ and HCO_3^-, and because HCO_3^- can function as a signalling molecule independent of pH (Chen *et al.* 2000), we investigated whether HCO_3^- contributes to RTN chemoreception.

Bicarbonate can modulate neuronal activity in a potentially pH-independent manner by activation of cyclic nucleotide signalling (Chen *et al.* 2000) or by influencing membrane potential by flux through Cl^- channels (Bonnet & Bingmann, 1993; Duran *et al.* 2010; Hamidi & Avoli, 2015) or electrogenic transporters (Romero & Boron, 1999). For example, HCO_3^- is a positive allosteric modulator of soluble adenylyl cyclase (Steegborn *et al.* 2005) that may contribute to central chemoreception including at the level of the RTN (Ritucci *et al.* 2005) or peripheral chemoreception (Summers *et al.* 2002) by activation of cAMP and protein kinase A (PKA) signalling. Interestingly, recent evidence showed that HCO_3^- stimulated hippocampal pyramidal neurons in a pH-independent manner by inhibition of KCNQ channels (Jones *et al.* 2014), possibly by PKA-dependent depletion of phosphatidylinositol 4,5-bisphosphate (PIP2; a requisite cofactor for channel function; Suh & Hille, 2008). Given that KCNQ channels are potent modulators of RTN chemoreceptor activity (Hawryluk *et al.* 2012; Hawkins *et al.* 2015; Mulkey *et al.* 2015), we therefore considered the possibility that HCO_3^- modulates RTN chemoreceptors by inhibition of KCNQ channels.

Here, we use a combination of slice-patch electrophysiology, pharmacology and intracellular pH imaging to show that HCO_3^-, in addition to H^+, can modulate activity of chemosensitive RTN neurons by mechanisms independent of pH, fast excitatory or inhibitory transmission, purinergic or nitric oxide signalling, soluble adenylyl cyclase (sAC) activity, or KCNQ channels. Conversely, CO_2/H^+-insensitive RTN neurons also did not respond to high HCO_3^-, suggesting HCO_3^- sensitivity is specific to respiratory chemoreceptors. These results establish HCO_3^- as a novel independent modulator of chemoreceptor activity.

Methods

Ethical approval

Animal use was in accordance with guidelines approved by the University of Connecticut Institutional Animal Care and Use Committee. Brain slices were isolated from neonatal Sprague-Dawley rat pups (7–12 days old; $n = 63$) (Charles River Laboratories, Kingston, NY, USA). All efforts were made to minimize the number of animals used.

Electrophysiological recordings in brainstem slices

Slices containing the RTN were prepared as previously described (Mulkey *et al.* 2004; Wenker *et al.* 2012). In short, rats were anaesthetized by administration of ketamine (375 mg/kg, I.P.) and xylazine (25 mg/kg, I.P.) and rapidly decapitated; brainstems were removed and transverse brain stem slices (300 μm) were cut using a microslicer (DSK 1500E; Dosaka, Kyoto, Japan) in ice-cold substituted Ringer's solution containing the following (in mM): 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose and 1 kynurenic acid. Slices were incubated for 30 min at 37°C and subsequently at room temperature in a normal Ringer's solution containing (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose. Both substituted and normal Ringer's solutions were bubbled with 95% O_2 and 5% CO_2 (pH 7.30).

Individual slices containing the RTN were transferred to a recording chamber mounted on a fixed-stage microscope with infrared Nomarski optics (Zeiss Axioskop FS); slices were perfused continuously (~2 ml/min) with a bath solution containing (in mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 Hepes and 10 glucose (equilibrated with 5% CO_2; pH 7.3). All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D converter, and recorded using pCLAMP 10.0 software (Molecular Devices, San Jose, CA, USA). Recordings were obtained at room temperature (~22°C) with patch electrodes pulled from borosilicate glass capillaries (Harvard Apparatus, Molliston, MA, USA) on a two-stage puller (P-97; Sutter Instrument, Novato, CA, USA) to a DC resistance of 5–7 MΩ when filled with a pipette solution containing the following (in mM): 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 Hepes, 10 EGTA, 3 Mg-ATP and 0.3 GTP-Tris (pH 7.20). Electrode tips were coated with Sylgard 184 (Dow Corning, Midland, MI, USA). Spontaneous neuronal activity was measured in the cell-attached voltage-clamp configuration with holding potential matched to the resting membrane potential of RTN neurons (V_{hold} = −60 mV) and with no current generated by the amplifier (I_{amp} = 0 pA) (Perkins, 2006). Firing rate histograms were generated by integrating action potential discharge in 10- to 20-s bins using Spike 5.0 software (Cambridge Electronic Design, CED, Cambridge, UK).

Intracellular pH imaging

In a subset of experiments, we measured intracellular pH (pH_i) of chemosensitive neurons during exposure to high CO_2 or HCO_3^-. After functionally identifying chemosensitive RTN neurons in the cell-attached configuration as described above, we obtained whole-cell access to dialyse cells with the pH-sensitive fluorescent dye BCECF free acid (Invitrogen, Carlsbad, CA, USA; dissolved in pipette solution at 100 μM). We waited ~15 min for BCECF to load the cell and establish stable fluorescence values before measuring pH_i. To activate the dye we toggled between (< 1 s transition) excitation wavelengths of 440 (pH-insensitive) and 490 nm (pH-sensitive) (xenon arc lamp), and the fluorescence emitted at 530 nm was collected for both excitation wavelengths. Images were acquired at an interval of 60 s. The background fluorescence intensity was subtracted, and an intensity ratio for each cell was calculated. The ratio of fluorescence intensities at 490 nm to that at 440 nm (B_{490}/B_{440}) was used to estimate pH_i. We normalized pH_i values measured in RTN neurons during incubation in 5% and 10% CO_2 to previously reported values (Ritucci *et al.* 2005). We also confirmed that all measured pH values are in line with expected ΔpH_i based on the following relationship:

$$\Delta pH_i = \Delta [CO_2] / \beta int$$

where β_{int} is intrinsic buffer power of 9.8 meq/l/pH unit (Nottingham *et al.* 2001) and $\Delta[CO_2]$ is the concentration difference in 5% (1.38 mM) and 10% (2.77 mM), determined using a CO_2 solubility coefficient of 0.03 mM/mmHg (Nottingham *et al.* 2001) and CO_2 partial pressures of 35.6 and 71.3 mmHg, respectively.

Solutions

Normal ACSF contained (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4 and 26 $NaHCO_3$, equilibrated with 10 glucose. To expose slices to hypercapnia, we equilibrated normal Ringer's solution with 10% CO_2 (pH 7.0). Isohydric HCO_3^- (which may also be considered isohydric hypercapnia) solution contained (in mM): 104 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 52 $NaHCO_3$ and 10 glucose, and was bubbled with 10% CO_2 (pH 7.3). HCO_3^--free Hepes buffer contained (in mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 Hepes and 10 glucose. For experiments where 26 mM $NaHCO_3$ was added to Hepes buffer, we lowered NaCl by an equimolar amount. The osmolarity of all solutions was maintained at ~300 mOsm.

Drugs

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. All drugs were bath applied at the following concentrations: XE991 (10 μM; Tocris Bioscience, Bristol, UK) was used to block KCNQ channels, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) was used to block AMPA/kainite receptors, strychnine (2 μM) was used to block glycine receptors, gabazine (10 μM) was used to block $GABA_A$ receptors,

pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, 5 μM) was used to block P2 receptors, acetazolamide (1 mM) was used to inhibit carbonic anhydrase activity, KH7 (10 μM; Tocris) was used to inhibit soluble adenylyl cyclase activity, and *N*-nitro-L-arginine methyl ester (L-NAME, 1 mM) was used to inhibit nitric oxide synthase activity.

Data analysis

Data are reported as mean ± SE. All statistical analysis was performed in GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons were made using a *t*-test or one-way ANOVA followed by Tukey or Dunnett multiple comparison tests as appropriate. The relevant values used for statistical analysis are provided in the Results.

Results

Chemosensitive RTN neurons were initially identified in slices incubated in normal Ringer's solution (HCO_3^- = 26 mM) based on their characteristic firing response to CO_2. Neurons that were spontaneously active in 5% CO_2 (pH_o 7.3) and responded to 10% CO_2 (pH_o ~7.0) with at least a 1.0 Hz increase in firing rate were considered chemosensitive. This level of CO_2/H^+ sensitivity is similar to what we (Wenker *et al.* 2012, Hawkins *et al.* 2015) and others (Ritucci *et al.* 2005) have previously reported for chemosensitive RTN neurons. Neurons that showed < 1 Hz firing response to 10% CO_2 or pH 7.0 were considered non-chemosensitive.

The goal of this study was to determine whether HCO_3^- can modulate activity of chemosensitive RTN neurons, and considering that these cells sense extracellular H^+ in part by activation of GPR4 (Kumar *et al.* 2015), we initially tested firing responses to low and high concentrations of HCO_3^- while maintaining pH_o. We found that exposure to HCO_3^--free Hepes-buffered solution (pH_o = 7.3 equilibrated with room air or 100% O_2) decreased activity of chemosensitive RTN neurons from 0.95 ± 0.01 to 0.20 ± 0.06 Hz ($F_{2,17}$ = 161.7, P < 0.0001) (Fig. 1*A*, *B*). Conversely, exposure to isohydric (pH_o = 7.3) HCO_3^- increased activity of chemosensitive RTN neurons from 0.48 ± 0.1 to 1.15 ± 0.2 Hz ($F_{2,18}$ = 186.1, P < 0.0001) (Fig. 1*C*, *D*). Note that basal activity in HCO_3^--free Hepes-buffered solution (0.1 ± 0.04 Hz) is less than basal activity in HCO_3^--buffered solution (26 mM; pH 7.3) (0.71 ± 0.08 Hz; T_{49} = 4.834, P < 0.0001), consistent with the possibility that HCO_3^- activates chemosensitive RTN neurons in a manner that contributes to baseline activity and CO_2/H^+ sensitivity. Bicarbonate sensitivity also appears specific to chemosensitive RTN neurons because CO_2/H^+-insensitive cells showed no measurable change in activity in response to isohydric HCO_3^- (P = 0.991) (Fig. 1*E*, *F*). The response of chemosensitive neurons to changes in HCO_3^- was retained in the presence of gabazine (10 μM) to block $GABA_A$ receptors, strychnine (2 μM) to block glycine receptors and CNQX (10 μM) to block AMPA/kainite receptors (Fig. 2*A*, *F*, *G*). These results confirm that neuronal firing responses to changes in HCO_3^- are not dependent on synaptic transmission or HCO_3^- flux through $GABA_A$ receptors (Bormann *et al.* 1987).

Based on evidence that KCNQ channels are key determinants of RTN chemoreceptor activity (Hawryluk *et al.* 2012) and in other brain regions HCO_3^- has been shown to activate sAC (Chen *et al.* 2000) and inhibit KCNQ by a mechanism that may involve PKA-dependent depletion of PIP2 (Jones *et al.* 2014), we also explored the possibility that KCNQ or sAC contribute to HCO_3^- modulation of RTN neurons. Contrary to our expectations, firing responses to changes in HCO_3^- were retained when KCNQ channels were blocked with XE991 (10 μM) (Fig. 2*B*, *F*, *G*) or when sAC activity was blocked with KH7 (10 μM) (Fig. 2*C*, *G*). For example, exposure to isohydric HCO_3^- increased chemoreceptor activity by 0.60 ± 0.03 Hz under control conditions, 0.69 ± 0.11 Hz in the presence of XE991 (Fig. 2B) and by 0.64 ± 0.11 after 10 min incubation in KH7 (Fig. 2*C*). It is also possible that exposure to high HCO_3^- in the presence of nitric oxide can facilitate the formation of peroxynitrite (Lymar *et al.* 1996), which can regulate neural activity by reduction–oxidation modulation of various ion channels and second messenger pathways. Therefore, we also tested the effects of isohydric HCO_3^- on RTN chemoreceptor activity when the activity of nitric oxide synthase was blocked with L-NAME. We found that incubation (20 min) in L-NAME (1 mM) had negligible effects on basal activity (Δ firing rate 0.20 ± 0.2 Hz; P = 0.281) and did not blunt the firing response to isohydric HCO_3^- (Δ firing rate 0.60 ± 0.1 Hz; P = 0.005) (Fig. 2*E*, *G*).

Considering that astrocytes contribute to RTN chemoreception by providing a CO_2/H^+-dependent purinergic drive to activate local neurons (Gourine *et al.* 2010; Huckstepp *et al.* 2010; Wenker *et al.* 2012), and because astrocytes express high levels of the Na^+/HCO_3^- cotransporter (NBC) (Erlichman & Leiter, 2010) and activation of the NBC has been shown to contribute to astrocyte chemoreception (Turovsky *et al.* 2016), we tested the possibility that HCO_3^--dependent activation of RTN neurons involves ATP release by RTN astrocytes. We found that exposure to isohydric HCO_3^- increases activity of RTN neurons by similar amounts under control conditions and during purinergic receptor blockade; exposure to a solution containing high CO_2 (10%) plus HCO_3^- (52 mM) with a pH_o of 7.3 increased chemoreceptor activity by 0.60 ± 0.03 Hz under control

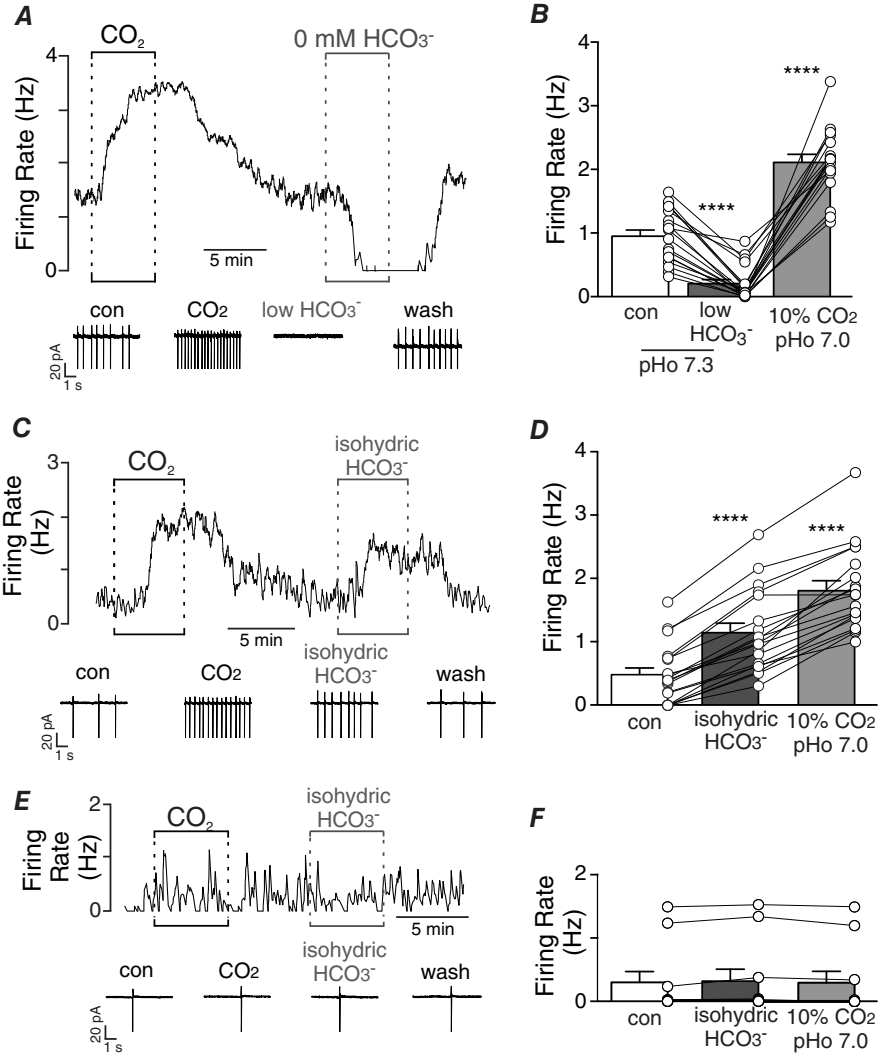

Figure 1. HCO_3^--dependent modulation of chemosensitive RTN neurons
A, trace of firing rate and segments of holding current shows a typical response of an RTN neuron in a slice incubated in normal Ringer's solution containing 26 mM HCO_3^- to an increase in CO_2 from 5% to 10%. After returning to control conditions, exposure to HCO_3^--free Hepes buffer (pH 7.3) strongly inhibited baseline activity. *B*, summary data ($n = 18$) show average firing rate under control conditions and during exposure to HCO_3^--free Hepes buffer (pH_o 7.3) and 10% CO_2 (pH_o 7.0). *C*, trace of firing rate and segments of holding current from a chemosensitive RTN neuron shows that exposure to isohydric HCO_3^- (10% CO_2/52 mM HCO_3^-) caused a robust increase in activity. *D*, summary data ($n = 19$) show average firing rate of chemosensitive RTN neurons under control conditions and during exposure to isohydric HCO_3^- and 10% CO_2 (pH_o 7.0). *E*, trace of firing rate and segments of holding current from a CO_2/H^+-insensitive RTN neuron shows that exposure to isohydric HCO_3^- (10% CO_2/52 mM HCO_3^-) minimally affected firing behaviour. *F*, summary data ($n = 10$) show average firing activity of CO_2/H^+-insensitive RTN neurons under control conditions and during exposure to isohydric HCO_3^- and 10% CO_2 (pH_o 7.0). One-way ANOVA with Tukey multiple comparison test. $^{****}P < 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com]

conditions and by 0.75 ± 0.10 Hz in the presence of PPADS (5 μM) (Fig. 2*D*, *G*).

A caveat to the above experiments is that manipulations of HCO_3^- may also affect pH_i (Table 1). For example, the transition from normal HCO_3^--buffered solution to a HCO_3^--free Hepes-buffered solution increased pH_i by 0.08 ± 0.02 pH units, presumably due to the rapid diffusion of CO_2 out of RTN neurons, whereas exposure to isohydric HCO_3^- decreased pH_i by 0.06 ± 0.01 pH units probably as a result of CO_2 influx. This is relevant because RTN neurons also express TASK-2 channels (Wang *et al.* 2013), which are activated by changes in intracellular or extracellular pH with a $pK_{1/2}$ of ∼8.6 (i.e. the pH that achieves half maximum channel activation) (Reyes *et al.* 1998; Cid *et al.* 2013). Our experimental conditions result in pH_i changes ∼1 pH unit lower than the effective pH_i sensing range of TASK-2. However, evidence also suggests TASK-2 channels contribute to RTN chemoreceptor activity at physiological pH of 7.3 and so even slight increases in pH_i, as observed during exposure to Hepes, may enhance TASK-2 activity, and thus inhibit neuronal firing. Consistent with this, in voltage clamp ($I_{hold} = -60$ mV, in tetrodotoxin to block neuronal action potentials), we found that exposure to HCO_3^--free Hepes buffer elicited a modest increase in outward current by activation of a relatively voltage-independent TASK-like current (data not shown). Because exposure to this experimental condition also results in a 0.08 ± 0.02 alkalization (Table 1), we suspect that pH rather than HCO_3^- activates this outward current. These results further support the possibility that activation of TASK-2 by intracellular alkalization can regulate activity of RTN neurons. However, these results also underline the need to independently control pH_i and HCO_3^-.

To differentiate between pH_i and HCO_3^--dependent modulation of neuronal activity, we tested effects of HCO_3^- (26 mM) in Hepes-buffered solution under control conditions and when carbonic anhydrase activity was blocked with acetazolamide (1 mM). Carbonic anhydrase catalyses the hydration/dehydration of CO_2

and so application of acetazolamide to a slice perfused with HCO_3^--ree Hepes solution is expected slow the formation of CO_2 during exposure to high HCO_3^-, and thus minimize changes in pH_i. To confirm this possibility, we included BCECF in our pipette internal solution to measure pH_i in chemosensitive RTN neurons during exposure to high HCO_3^- in Hepes alone and in Hepes solution supplemented with acetazolamide. Exposure to high HCO_3^- alone caused a modest acidification of 0.07 ± 0.01 pH units ($T_3 = 12.63$, $P < 0.01$) that persisted for the 5 min duration of the exposure (Fig. 3*A*, *B*). Application of acetazolamide (1 mM) also decreased pH_i by 0.40 ± 0.085 pH units ($T_2 = 4.714$, $P<0.05$) (Fig. 3*A*), probably by favouring the intracellular accumulation of

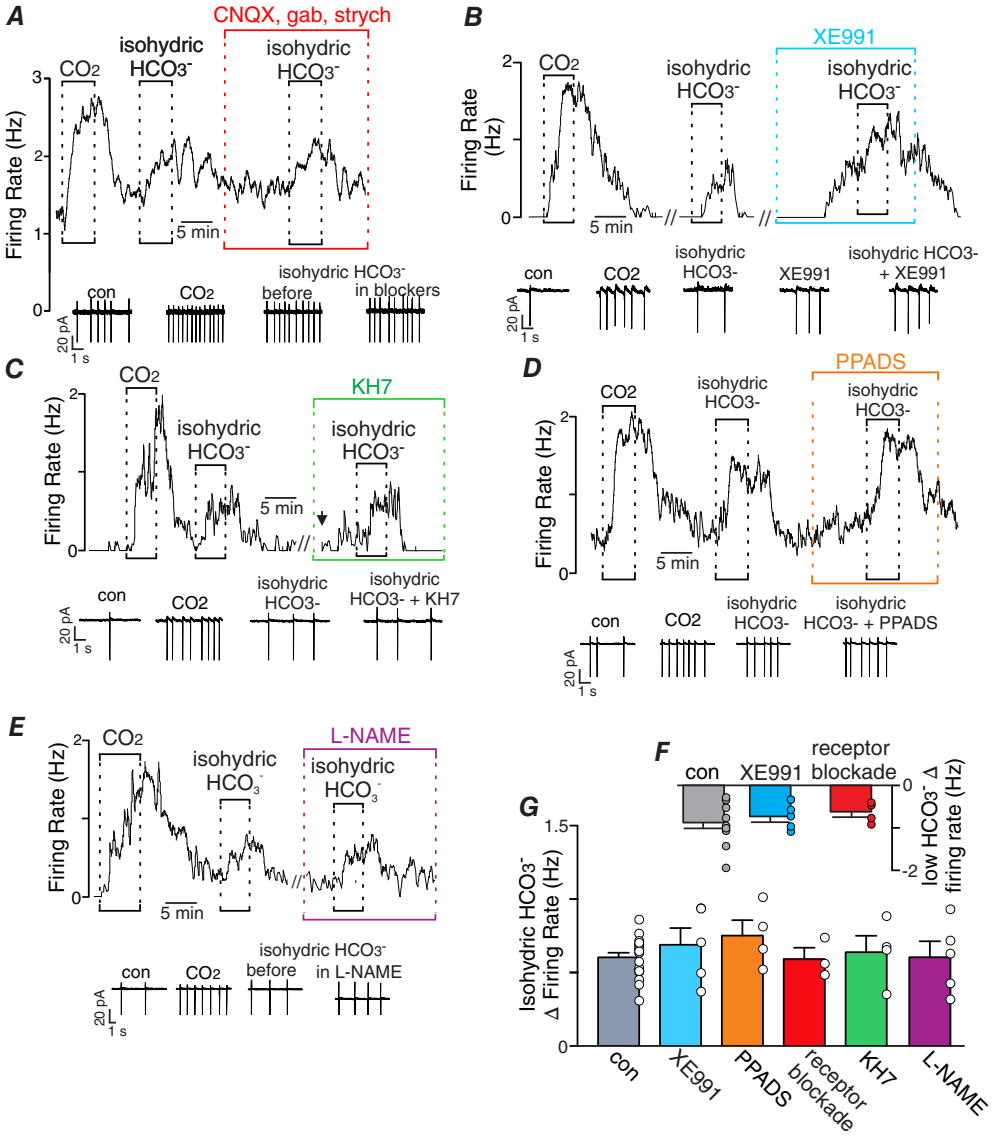

Figure 2. HCO_3^- directly modulates activity of RTN neurons
A–E, traces of firing rate and segments of holding current from chemosensitive RTN neurons show that exposure to isohydric HCO_3^- (10% CO_2/52 mM HCO_3^-) stimulated neural activity under control conditions and after 10 min of incubation in a transmitter receptor blocker cocktail containing CNQX (10 μM), gabazine (10 μM) and strychnine (2 μM) (*A*), KCNQ channels were blocked with XE991 (10 μM) (*B*), sAC activity was inhibited with KH7 (10 μM) (*C*), purinergic receptors were blocked with PPADS (5 μM) (*D*), and when nitric oxide synthase was inhibited with L-NAME (1 mM). *F*, summary data show that exposure to HCO_3^--free Hepes buffer inhibited activity under control conditions ($n = 10$), and in XE991 ($n = 5$) or the blocker cocktail ($n = 4$). *G*, summary data plotted as isohydric HCO_3^--induced change in activity under control conditions ($n = 13$) and in the presence of XE991 ($n = 5$), PPADS ($n = 4$), blocker cocktail ($n = 3$), KH7 ($n = 4$), or L-NAME ($n = 5$). //, a 10–20 min break in the recording. ↓, injection of a positive DC current to adjust baseline activity to near control levels. One-way ANOVA ($F_{5,33} = 0.5909$, $P > 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. pHi and firing responses of RTN neurons under all experimental conditions

Test condition	ΔpH_i	Δ firing rate (Hz)
10% CO_2	0.12 acidification‡	1.25 ± 0.1*
HCO_3^--free Hepes	0.08 ± 0.02 alkalization*	−0.75 ± 0.1*
high HCO_3^- (52 mM) Ringer's solution	0.06 ± 0.01 acidification*	0.66 ± 0.1*
Hepes + HCO_3^- (26 mM)	0.07 ± 0.01 acidification*	0.73 ± 0.1*
Hepes + HCO_3^- (26 mM) + acetazolamide	0.09 ± 0.01 alkalization*	0.72 ± 0.1*

‡5 and 10% CO_2 were used as pH_i calibration points.*Significant differences from control ($P < 0.05$)

metabolically generated carbonic acid (Erlichman *et al.* 1994). In the continued presence of acetazolamide, a second exposure to high HCO_3^- this time increased pH_i by 0.09 ± 0.01 pH units ($T_2 = 5.883$, $P < 0.05$), suggesting that in this condition CO_2 transport is reduced and HCO_3^- is the primary molecule transported into the cell (Fig. 3*A*, *B*).

These results show that exposure to HCO_3^- alone and in the presence of acetazolamide have opposite effects of pH_i, and assuming TASK-2 channels are the sole pH_i

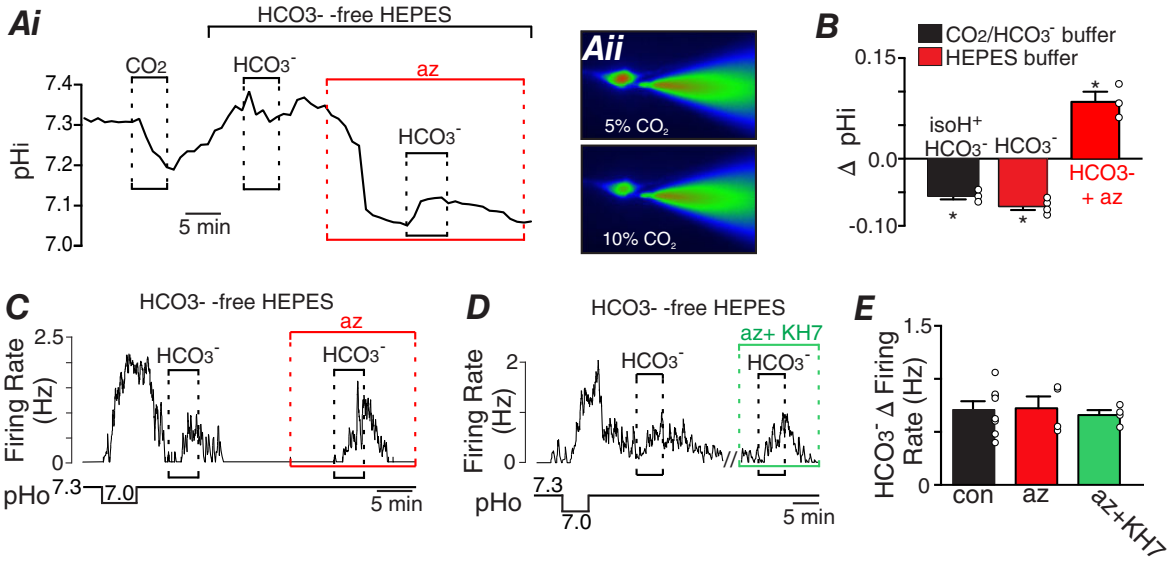

Figure 3. HCO_3^- modulates activity of chemosensitive RTN neurons by a mechanism independent of pH_i

BCECF (100 μM) was included in our pipette internal solution to measure pH_i of chemosensitive RTN neurons incubated in Hepes buffer during exposure to high HCO_3^- (26 mM) alone and in the presence of acetazolamide (az; 1 mM). *A*, trace of pH_i (*Ai*) and fluorescence images (490 nm excitation) (*Aii*) from a chemosensitive RTN neuron in normal Ringer's solution (26 mM HCO_3^-) equilibrated with 5% CO_2 (pH_o 7.3) shows that exposure to 10% CO_2 decreased pH_i ~0.1 pH units. After returning to control conditions exposure to HCO_3^--free Hepes buffer (pH_o 7.3) increased pH_i ~0.08 pH units. In the continued presence of Hepes buffer, exposure to HCO_3^- (26 mM) reversibly decreased pH_i by ~0.07 pH units under control conditions. Under these conditions exposure to acetazolamide (1 mM) also decreased pH_i by ~0.4 pH units. However, in acetazolamide subsequent exposure to HCO_3^- (26 mM) this time increased pH_i ~0.1 pH units. *B*, summary data plotted as change in pH_i during exposure to isohydric HCO_3^- (10% CO_2 + 52 mM HCO_3^-) in normal Ringer's solution ($n = 4$), and HCO_3^- (26 mM) alone ($n = 3$) or together with acetazolamide under Hepes buffer conditions ($n = 3$). *C*, trace of firing rate shows a typical H^+ response of an RTN neuron in a slice incubated in Hepes buffer. After returning to control conditions (pH– 7.3), exposure to HCO_3^- (26 mM; pHo 7.3) alone or in the presence of acetazolamide (1 mM) increased ~0.75 Hz. Note that bath application of acetazolamide minimally affected neuronal activity despite resulting in a strong intracellular acidification. *D*, trace of firing rate from a chemosensitive RTN neuron in a slice incubated in Hepes buffer shows that exposure to HCO_3^- (26 mM; pH_o 7.3) increased neural activity by similar amounts under control conditions and in the presence of acetazolamide (1 mM) plus KH7 (10 μM). *E*, summary data show that the firing response to HCO_3^- was similar under control conditions ($n = 7$) and in acetazolamide alone ($n = 4$) or in combination with KH7 ($n = 4$) (one-way ANOVA; $F_{2,13} = 0.115$, $P > 0.05$). *Paired *t* test, $P < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

sensor in these cells, we expect these changes in pH_i to also have opposite effects on neural activity. Therefore, by comparing neuronal responses to HCO_3^- under these conditions, we will be able to determine whether HCO_3^- signalling affects chemoreceptor function independent of pH_i. We found that chemosensitive RTN neurons in slices incubated in HCO_3^--free Hepes buffer at pH 7.3 showed lower baseline activity (0.14 ± 0.06 Hz) compared to RTN neurons in slices incubated in HCO_3^--buffered conditions at the same pH (0.79 ± 0.09 Hz) ($T_{39} = 3.864$, $P < 0.001$), suggesting factors other than pH influence chemoreceptor activity. Consistent with this, we found under Hepes buffer conditions that exposure to HCO_3^- (26 mM) alone increased activity of chemosensitive RTN neurons by 0.71 ± 0.08 Hz ($T_7 = 9.1$, $P < 0.0001$) (Fig. 3*C*). However, bath application of acetazolamide (1 mM) minimally affected chemoreceptor activity (Fig. 3*C*) despite causing a large intracellular acidification (Fig. 3*A*). These results suggest that TASK-2 channels are minimally active at pH values less than 7.3. Furthermore, in the continued presence of acetazolamide, a second exposure to 26 mM HCO_3^- increased chemoreceptor activity by 0.72 ± 0.11 Hz ($T_3 = 14.98$, $P < 0.001$) (Fig. 3*C–E*), despite occurring in conjunction with an 0.09 ± 0.01 increase in pH_i, which is expected to limit chemoreceptor activity by TASK-2 channel activation. These results suggest HCO_3^- can regulate activity of RTN neurons independent of pH_i. Additionally, the combined application of acetazolamide plus KH7 (10 μM) minimally affected HCO_3^--mediated excitation (Fig. 3*D*, *E*), suggesting sAC activity does not contribute to HCO_3^- modulation of RTN neurons.

Discussion

The main finding of this study is that HCO_3^-, in addition to H^+, can selectively activate chemosensitive RTN neurons. This is important because identifying all relevant signalling molecules is essential for understanding how chemoreceptors function, and as the levels of HCO_3^- along with other chemosensory modalities (namely H^+) are buffered by independent cellular mechanisms (Chesler, 2003), these results suggest HCO_3^- chemoreception adds additional information regarding changes in CO_2 that are not necessarily reflected by pH. Furthermore, HCO_3^- may be particularly important for regulating activity of RTN chemoreceptors during sustained intracellular acidifications when TASK-2 channels, which appear to be the sole intracellular pH sensor, are minimally active. In addition, considering CO_2/H^+-induced changes in HCO_3^- may be small relative to the background HCO_3^- (26 mM), it is also possible that the role of HCO_3^- is to provide a tonic enhancement of baseline activity and CO_2/H^+ sensitivity.

Mechanisms of RTN chemoreception

Chemosensitive RTN neurons primarily sense extracellular H^+ by inhibition of TASK-2 channels (Wang *et al.* 2013) and activation of GPR4 (Kumar *et al.* 2015). Although TASK-2 channels also sense pH_i, their capacity to respond to pH_i changes in the lower range is limited because TASK-2 channels have a pKa of 7.8 (Reyes *et al.* 1998). Consistent with this, our results show that intracellular acidification by bath application of acetazolamide minimally affected neural activity. However, under these conditions RTN neurons are still able to respond to HCO_3^-. These results show that HCO_3^- chemoreception expands the functional pH_i sensing capacity of RTN neurons.

The CO_2/H^+-dependent output of RTN neurons is further enhanced by CO_2/H^+-evoked ATP-purinergic signalling by local astrocytes (Gourine *et al.* 2010; Wenker *et al.* 2010, 2012), which activates chemosensitive neurons directly (Gourine *et al.* 2010; Wenker *et al.* 2012) and indirectly by maintaining vascular tone when CO_2/H^+ levels are high (Hawkins *et al.* 2017). Interestingly, the contribution of astrocytes to RTN chemoreception requires both H^+ and molecular CO_2; exposure to H^+ has been shown to inhibit astrocyte Kir4.1 potassium K^+ channels (Wenker *et al.* 2010) and activate the NBC which together favour Ca^{2+} influx by reverse mode operation of the sodium calcium exchanger (NCX) (Turovsky *et al.* 2016). However, CO_2-dependent gating of connexin 26 hemichannels also appears to be required for ATP release (Huckstepp *et al.* 2010). Because HCO_3^- did not facilitate purinergic modulation of RTN neurons, we do not think this signalling pathway contributes to HCO_3^- chemoreception.

Possible mechanisms of HCO_3^- chemoreception

In comparison to pH, HCO_3^- has been shown to target only a limited number of effectors. For example, olfactory neurons sense CO_2 in part by HCO_3^- activation of guanylyl cyclase-D (GC-D) (EC_{50} ~20 mM; Tresguerres *et al.* 2010) which stimulates neuronal activity by activation of cyclic nucleotide-gated channels (Hu *et al.* 2007). Although, GC-D has only been found in neurons localized to the olfactory bulb (Fulle *et al.* 1995), because the olfactory and respiratory systems are functionally coupled and may share information regarding timing of inspiration and expiration (Perez de Los Cobos Pallares *et al.* 2016; Short *et al.* 2016), it is conceivable that chemosensitive RTN neurons share common HCO_3^- sensing mechanisms including GC-D. However, this interesting possibly requires further investigation. Furthermore, despite evidence that HCO_3^- can enhance the production of peroxynitrite (Lymar *et al.*

1996), which can modulate neural function by oxidizing nucleophilic residues on target proteins including various ion channels, blocking the production of nitric oxide did not affect HCO_3^- chemosensitivity. These results argue against the possible involvement of peroxynitrite in HCO_3^- signalling. In addition, our evidence that HCO_3^- modulation of RTN neurons was retained when $GABA_A$ receptors were blocked rules out involvement of this Cl^- channel. However, involvement of other Cl^- channels remains an open possibility. It is also possible that HCO_3^- directly interacts with other unidentified ion channels.

In summary, we show that variations in HCO_3^- above and below normal physiological levels (26 mM) increase and decrease activity of chemosensitive RTN neurons by mechanisms independent of pH, fast excitatory or inhibitory transmission, purinergic or nitric oxide signalling, sAC activity, or KCNQ channels. Although the mechanism(s) of HCO_3^- chemoreception remain unknown, these results establish HCO_3^- as a novel independent modulator of chemoreceptor activity.

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Additional Information

Competing interests

We have no competing interests.

Author contributions

CMG: experimental design; collection and analysis of *in vitro* data; revising the manuscript; final approval of the manuscript. DKM: experimental design; data analysis; revising the manuscript; drafting the manuscript; final approval of the manuscript.

Funding

This work was supported by funds from the National Institutes of Health Grants HL104101 and R01HL137094. Additional funds were also provided by the Dravet Foundation.