Recruitment of excitatory serotonergic neurotransmission to cardiac vagal neurons in the nucleus ambiguus post hypoxia and hypercapnia


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Kamendi HW, Cheng Q, Dergacheva O, Frank JG, Gorini C, Jameson HS, Pinol RA, Wang X, Mendelowitz D. Recruitment of excitatory serotonergic neurotransmission to cardiac vagal neurons in the nucleus ambiguus post hypoxia and hypercapnia. J Neurophysiol 99: 1163–1168, 2008. First published January 9, 2008; doi:10.1152/jn.01178.2007. Inhibitory GABAergic and glycnergic neurotransmission to cardioinhibitory cardiac vagal neurons (CVNs) increase during inspiratory activity and likely mediate respiratory sinus arrhythmia, while the frequency of excitatory postsynaptic currents (EPSCs) in CVNs are unaltered during the different phases of respiration. However, following hypoxia and hypercapnia (H/H), the parasympathetic activity to the heart increases and thus far, identification of the pathways and neurotransmitters that are responsible for exciting CVNs post H/H are unclear. This study identifies different excitatory pathways to CVNs recruited post H/H. Spontaneous and inspiratory-related EPSCs were recorded in CVNs before, during, and after 10 min of H/H in an in vitro slice preparation that retains rhythmic respiratory activity. Before and during H/H, EPSCs in CVNs were completely blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and d-(−)-2-amino-5-phosphonopentanoic acid (AP5), selective AMPA/kainate and N-methyl-D-aspartate (NMDA) receptor blockers, respectively. However, after H/H, there was a significant increase in EPSCs during each inspiratory burst. While some of the inspiratory-related EPSCs were blocked by the broad purinergic receptor antagonist pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS) and the specific P2X receptor antagonist 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate monolithium trisodium salt (TNP-ATP) a P2X receptor blocker, most of the recruited excitatory neurotransmission to CVNs is serotonergic because odansetron, a activating P2X and 5-HT3 receptors, respectively, are recruited to excite CVNs in the post H/H recovery period.

INTRODUCTION

Heart rate is strongly influenced by the changing phases of normal respiration. Heart rate increases with inspiration and decreases with expiration, and this occurs in large part due to interactions between respiratory neurons and parasympathetic cardiac vagal neurons (CVNs) in the brain stem. This respiratory sinus arrhythmia (RSA) of CVNs is primarily mediated by inspiratory-evoked increases in inhibitory GABAergic and glycnergic synaptic inputs with no change in excitatory glutamatergic synaptic inputs to CVNs (Evans et al. 1998). However, whereas RSA occurs under normal conditions, exposure to environmental insults such as hypoxia and/or hypercapnia can dramatically alter cardiorespiratory function, including the inspiratory-related modulation of CVN activity (Evans et al. 2005; Griffioen et al. 2007; Huang et al. 2006, 2007; Neff et al. 2003). Responses to hypoxia and hypercapnia (H/H) include generalized vasodilation and increased cutaneous temperature that are mediated by the sympathetic nervous system (Simmons et al. 2007). In addition, H/H elicits tachycardia at the onset of the H/H that is followed by bradycardia as the H/H episodes continue (Bonsignore et al. 1994). The biphasic tachycardia followed by bradycardia during H/H is likely partly due to the biphasic increase then decrease in inhibitory GABAergic and glycnergic inputs to CVNs (Neff et al. 2003). On recovery from H/H, when normal levels of oxygen and carbon dioxide are restored, increased vagal tone and bradycardia are maintained (Pichot et al. 2000; Roche et al. 2002). The mechanisms responsible for the post hypoxic bradycardia are still not clearly understood.

Neurons in the brain stem responsible for chemosensitivity and initiating cardiorespiratory responses to H/H have not been fully elucidated but likely candidates include ATP (Gourine 2005; Gourine et al. 2005), and glutamate-containing neurons of the retrotapezoid nucleus (Guyenet et al. 2005) as well as glutamate- and acetylcholine-containing neurons in the arcuate nuclei (Benarroch et al. 2001, 2007) and the serotonin-rich medullary raphe neurons (Richerson et al. 2005). Chemoreceptive nuclei could potentially mediate changes in heart rate by activating direct pathways from these neurons to CVNs in the nucleus ambiguus or via polysynaptic pathways. Measurements of neurotransmitters released within the ventral respiratory group (VRG), an area located close to CVNs, in response to hypoxia show transient increases in GABA and glutamate after induction of hypoxia (Richter et al. 1999). As hypoxia progresses, serotonin and adenosine 5′-triphosphate (ATP) levels are released at low levels and the levels of serotonin and ATP peak and remain elevated after termination of the hypoxic episode (Richter et al. 1999). P2X receptors have been also shown to be involved in the responses to hypoxia in CVNs (Griffioen et al. 2007), carotid body (Lahiri et al. 2007), and rostral ventrolateral medulla (RVLM) neurons (Thomas et al. 1999). Furthermore, P2X2-deficient mice exhibit a diminished response to hypoxia (Rong et al. 2003). The goals of this study were to identify the excitatory pathways recruited and respon-
sible for the excitation of CVNs post H/H. More specifically, the role of glutamate, 5-HT and purinergic pathways to CVNs were examined before, during, and in recovery from H/H.

METHODS

Fluorescent labeling of CVNs and medullary slice preparation

Neonatal Sprague-Dawley rats (P3–P7; Hilltop, Scottsdale, PA) were anesthetized and cooled to ~4°C to slow the heart rate. A right thoracotomy was performed, and the retrograde fluorescent tracer X-phodamine-5-(and-6)-isothiocyanate (Molecular Probes, Eugene, OR) was injected into the fat pads at the base of the heart. After 24–48 h of recovery, animals were anesthetized with isoflurane and killed by cervical dislocation, and the brain tissue was placed in a 4°C physiologic saline solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 5 glucose, and 10 HEPES bubbled with 100% O₂ (pH 7.4). All animal procedures were performed with the approval of the Animal Care and Use Committee of The George Washington University in accordance with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and the National Institutes of Health publication, “Guide for the Care and Use of Laboratory Animals.”

The medulla was removed with care to preserve the hypoglossal cranial nerve rootlet and was mounted on a cutting block and placed into a recording chamber that allowed perfusion (4 ml/min) above of the tissue. A single thick (770 – 870 μm) section that included inferior olives and the NA could be visualized on the rostral surface cranial nerve rootlet and was mounted on a cutting block and placed in physiologic saline solution containing (in mM) 135 K-gluconic acid, 10 HEPES, 10 EGTA, 1 CaCl₂, and 1 MgCl₂, at a pH of 7.3.

H/H

Rhythmic inspiratory-related and spontaneous excitatory postsynaptic currents (EPSCs) in a single CVN were recorded simultaneously for 4 min (control period) in ACSF equilibrated with 95% O₂-5% CO₂. Slices were then perfused with H/H ACSF (equilibrated with 85% N₂-6% O₂-9% CO₂) for 10 min and then returned to the original perfuse for ±60 min during which different drug regimens (see focal drug application in the following text) were applied to isolate the excitatory inputs recruited to CVNs in the post H/H period.

Focal drug application

All the drugs used in these experiments were applied using a pneumatic picopump pressure delivery system (WPI, Sarasota, FL). Drugs were ejected from a patch pipette positioned within 30 μm from the patched CVN. The maximum range of drug application has been previously determined to be 100–120 μm downstream from the drug pipette and considerably less behind the drug pipette (Wang et al. 2001). Drugs used included ondansetron (100 μM) to block 5-HT3 receptors, pyridoxalphosphate-6-azophenyl-2', 4'-disulfophonic acid (PPADS, 100 μM) and 2,3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP, 100 μM) to block purinergic and P2X receptors, respectively, and finally 6-cyano-7-nitroquinoline-2,3-dione (CNQX, 50 μM) and (−)-2-amino-5-phosphono pentanoic acid (AP5, 50 μM) were used to block excitatory glutamatergic neurotransmission to CVNs. All drugs were obtained from Sigma Chemical (St. Louis, MO).

Recording respiratory network activity

The thick medullary slice preparation generates rhythmic inspiratory-related motor discharge in hypoglossal cranial nerve fibers (Smith et al. 1991). Spontaneous respiratory-related activity was recorded by monitoring motor-neuron population activity from hypoglossal nerve rootlets using a suction electrode. Hypoglossal rootlet activity was monitored motor-neuron population activity from hypoglossal nerve rootlets using a suction electrode. Hypoglossal rootlet activity was monitored during control conditions. The frequencies and amplitudes of EPSCs in CVNs were not altered by inspiratory bursts, see Fig. 1. Similarly the frequency and amplitude of EPSCs was not altered by ondansetron (100 μM) to block 5-HT3 receptors, pyridoxalphosphate-6-azophenyl-2', 4'-disulfophonic acid (PPADS, 100 μM) and 2,3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP, 100 μM) to block purinergic and P2X receptors, respectively, and finally 6-cyano-7-nitroquinoline-2,3-dione (CNQX, 50 μM) and (−)-2-amino-5-phosphonopentanoic acid (AP5, 50 μM) were used to block excitatory glutamatergic neurotransmission to CVNs. All drugs were obtained from Sigma Chemical (St. Louis, MO).

Data analysis

Synaptic events were detected using MiniAnalysis version 5.6.12 (Synaptosoft, Decatur, GA). The frequency of EPSCs that occurred in CVNs were grouped into 1-s bins and cross-correlated with the onset of inspiratory-related hypoglossal activity. Data were analyzed from all bursts during the last 2 min of the control period, during the last 2 min of the 10-min period of hypoxia/hypercapnia, and from minutes 6 – 8 during each 8-min drug regimen application period. In addition at the end of the experiment, the last 2 min recorded during the drug-free period was analyzed. These periods were chosen for analysis because during these periods, H/H-evoked changes and drug application had reached a steady state. Statistical comparisons were performed within a condition using a one-way ANOVA with repeated measures, and a two-way ANOVA with repeated measures was utilized to examine the differences between the spontaneous and respiratory-related EPSCs in response to various time-dependent or drug application periods. For the two-way ANOVA with repeated measures, comparisons between drug applications or time periods the 5 s of spontaneous EPSC frequency before the inspiratory burst was averaged into a single spontaneous frequency value. Significant difference was set at P < 0.05.

RESULTS

5-HT3 and purinergic receptors do not mediate excitation of CVNs during normal respiration

Previous studies have shown that under control conditions CVNs receive spontaneous glutamatergic EPSCs because AP5 and CNQX, NMDA and non-NMDA receptor blockers, respectively, block most, if not all, excitatory inputs to CVNs (Evans et al. 2005; Neff et al. 1998). We tested the hypothesis that CVNs also receive 5-HT3 and purinergic receptor signaling during control conditions. The frequencies and amplitudes of EPSCs in CVNs were not altered by inspiratory bursts, see Fig. 1. In addition, there was no change in the frequency of EPSCs from spontaneous 4.2 ± 0.1 Hz to inspiratory period 4.2 ± 0.2 Hz (n = 6, P > 0.05) see Fig. 1. Similarly the frequency and amplitude of EPSCs was not altered by ondansetron (100 μM) or PPADS (100 μM). However, AP5 and CNQX, NMDA
and non-NMDA receptor blockers, abolished all EPSCs, (4.2 ± 0.1 to 0.6 ± 0.3 n = 6, P < 0.05, see Fig. 1). The block of EPSCs with AP5 and CNQX was reversible, as shown in Fig. 1.

Inspiratory-related cardiac vagal neuron responses to hypoxia and hypercapnia and during recovery

Inspiratory bursting frequency and duration significantly (P < 0.05) decreased during H/H (from 3.2 ± 0.4 to 2.0 ± 0.2 bursts/min and 3.3 ± 0.3 to 1.3 ± 0.1 s, respectively), and in recovery both inspiratory frequency and duration significantly (P < 0.05) increased (from 2.0 ± 0.2 to 4.2 ± 0.3 bursts/min and 1.3 ± 0.1 to 12.8 ± 3.2 s, respectively). These changes persisted for the duration of these experiments (≤60 min).

As reported previously CVNs do not receive any inspiratory-related excitation during control or 10 min of hypoxia and hypercapnia (Huang et al. 2007). However during recovery from H/H, there was a significant increase in the frequency of inspiratory related EPSCs in CVNs, see Fig. 2. The EPSC frequency increased from 5.4 ± 0.4 to 11.0 ± 0.2 Hz (n = 9, P > 0.05) during inspiratory activity. The increase in excita-

![Image](https://via.placeholder.com/150)

**Fig. 1.** 5-HT3 and P2X receptor antagonists do not mediate excitation of cardiac vagal neurons (CVNs) during control respiratory activity. Inspiratory-related bursting activity was recorded from the hypoglossal rootlet (XII) and electronically integrated (top). Fluorescently identified CVNs were patch clamped in the whole cell configuration, and glutamatergic neurotransmission was isolated by focal application of GABAergic (gabazine; 25 μM) and glycinergic (strychnine; 1 μM) receptor antagonists in this and all subsequent figures. Ondansetron (100 μM), 100 μM pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS), and 25 μM CNQX/1 μM AP5 were applied sequentially to CVNs with a 5-min period between applications. A typical experiment is shown in A, and the average results from 6 CVNs are illustrated in B. There was no significant difference in excitatory postsynaptic current (EPSC) frequency or amplitude among the control, ondansetron, and PPADS applications. In the presence of 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX) and D(-)-2-amino-5-phosphonopentanoic acid (AP5), EPSC events were blocked, *P < 0.001.

**Fig. 2.** Hypoxia/hypercapnia (H/H) evokes a long-lasting inspiratory-related excitation in CVNs. Changing the perfusate from artificial cerebrospinal fluid (ACSF) equilibrated with 95% O2-5% CO2 to ACSF equilibrated with 9% CO2, 6% O2, 85% N2, H/H, did not alter the frequency of EPSCs to CVNs. However, H/H induced an increase in the frequency of EPSCs to CVNs during recovery from H/H, which lasted for ≤48 min. * denotes a statistically significant difference of P < 0.0001 between spontaneous and respiratory evoked EPSC frequency using a 1-way ANOVA with repeated measures in this and all subsequent figures.
tory inputs to CVNs during recovery persisted for \( \leq 1 \) h after termination of the H/H period, see Fig. 2 (10.1 \( \pm \) 0.7 Hz at 6–8 min and 11.1 \( \pm \) 0.3 Hz at 46–48 min).

**NMDA and AMPA receptor antagonists do not diminish the inspiratory-related excitation of CVNs**

To test the hypothesis that the increase in inspiratory-related excitation post H/H was glutamatergic, CNQX and AP5 were applied. Surprisingly application of AP5 and CNQX did not significantly alter (11.1 \( \pm \) 0.3 to 10.8 \( \pm \) 0.5 Hz \( n = 10, \) \( P > 0.05 \)) the excitatory pathway to CVNs recruited post H/H, see Figs. 3 and 4.

**5-HT3 receptors mediate the spontaneous and inspiratory-related excitation of CVNs**

Because both purinergic and 5-HT neurons can mediate chemosensitivity and pH signaling within the brain stem (Ling et al. 2001), and 5-HT fibers are known to make axo-somatic connections with CVNs (Izzo et al. 1993; Takeuchi et al. 1983), we hypothesized that either purinergic or 5-HT pathways acting via P2 or 5-HT3 receptors, respectively, could mediate inspiratory-related excitation of CVNs post H/H. To test these hypotheses, PPADS and ondansetron were added sequentially by focal application. PPADS significantly inhibited, but did not eliminate, the inspiratory-related EPSCs from 11.6 \( \pm \) 0.2 to 8.4 \( \pm \) 0.3 Hz \( n = 8, \) \( P < 0.05 \)), see Fig. 3A. Ondansetron subsequently completely blocked the increase in inspiratory-related excitation of CVNs (inspiratory; from 8.4 \( \pm \) 0.3 to 2.7 \( \pm \) 0.5 Hz \( n = 8, \) \( P < 0.05 \)), see Fig. 3A, and in addition, decreased the spontaneous EPSCs (5.4 \( \pm \) 0.4 to 2.69 \( \pm \) 0.5 Hz \( n = 8, P < 0.05 \)) see Fig. 3B. The inhibition by PPADS and ondansetron were reversible.

**TNP-ATP a P2X receptor antagonist significantly inhibits inspiratory-related excitation during recovery from hypoxia and hypercapnia**

In another set of experiments, PPADS was substituted with TNP-ATP, a more specific P2X antagonist (Gever et al. 2006) and ondansetron was applied before TNP-ATP. Similar to the previous series of experiments, CNQX and AP5 did not significantly diminish the inspiratory-related excitation of CVNs (10.1 \( \pm \) 0.5 to 9.9 \( \pm \) 0.6 Hz \( n = 9, \) \( P > 0.05 \)) post H/H, see Fig. 4A. Ondansetron inhibited both the inspiratory related (9.9 \( \pm \) 0.6 to 5.8 \( \pm \) 0.5 Hz \( n = 10, \) \( P < 0.05 \)) see Fig. 4A and spontaneous EPSCs (5.9 \( \pm \) 0.6 to 1.9 \( \pm \) 0.7 Hz \( n = 10, \) \( P < 0.05 \)) in CVNs post H/H, see Fig. 4B. Addition of TNP-ATP sequentially by focal application.

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**FIG. 3.** Activation of 5-HT3 receptors mediates respiratory-related EPSC neurotransmission to CVNs during recovery from hypoxia/hypercapnia. CNQX, AP5, PPADS, and ondansetron were applied sequentially to characterize the excitatory synaptic inputs to CVNs. A typical experiment from an unexposed animal is shown at the top, A, and the average data from 10 cells is shown in the histograms, A, bottom. AP5 and CNQX did not significantly alter respiratory-related excitatory neurotransmission to CVNs during recovery from H/H. Rather, PPADS, a broad purinergic antagonist, inhibited the inspiratory-related excitation while ondansetron, the 5-HT3 receptor blocker, inhibited both the spontaneous and inspiratory-related EPSCs. The inhibition by ondansetron and PPADS was reversible. \# and \( \square \) denotes a statistically significant difference of \( P < 0.0001 \) between respiratory-related EPSC frequency in a period compared with control values using a 2-way ANOVA with repeated measures in this and all subsequent figures. + denotes statistically significant difference of \( P < 0.001 \) using a 2-way ANOVA with repeated measures comparing the spontaneous EPSC frequency in the control and during each drug application in this and all subsequent figures.
abolished the inspiratory-related EPSCs from (5.8 ± 0.5 to 1.9 ± 0.5 Hz; n = 10, P < 0.05), Fig. 4A, with no further significant change to spontaneous EPSCs, see Fig. 4B. The inhibition by TNP-ATP and ondansetron were reversible, (inspiratory 1.9 ± 0.5 to 10.87 ± 0.77 Hz, n = 8 P < 0.05 and spontaneous 1.9 ± 0.7 to 5.3 ± 0.7 Hz n = 9 P < 0.05), see Fig. 4.

**DISCUSSION**

In this study, we sought to determine the synaptic mechanisms that are involved in exciting CVNs following hypoxia and hypercapnia. The findings of this study are 1) purinergic P2X and serotonergic 5-HT3 receptors do not mediate excitation of CVNs during normal inspiratory activity. 2) A long-lasting inspiratory-related excitation to CVNs is recruited during recovery from H/H, which is not mediated by glutamatergic neurotransmission. 3) Following hypoxia and hypercapnia, both spontaneous EPSCs and the inspiratory-related increase in EPSCs are mediated by the activation of 5-HT pathway, and in addition, during inspiration a purinergic pathway is also recruited that activates P2X receptors in CVNs.

Previous studies from this lab have demonstrated that under control conditions EPSCs in CVNs are primarily mediated by glutamate via NMDA and non-NMDA receptors (Neff et al. 1998). For example, a likely link in the baroreflex arc is the glutamate pathway evoked on stimulation of the NTS (Neff et al. 1998) as well as vagal afferent fibers (Evans et al. 2003). Similar to previously reported results CVNs do not receive inspiratory-related excitation during normal respiration (Evans et al. 2005; Huang et al. 2007). This study confirms that excitatory 5-HT3 and P2 receptors are not spontaneously active in CVNs under control conditions.

However H/H significantly alters cardiorespiratory function. The results in this study demonstrate a long-lasting (≥1 h) increase in inspiratory-related excitation to CVNs following an acute 10-min period of hypoxia and hypercapnia. It is possible this post H/H augmentation of inspiratory-related EPSCs is elicited by long-term facilitation (LTF) of the respiratory network resulting from exposure to hypoxia and/or hypercapnia. Respiratory LTF is mediated by modulation of the respiratory network by both serotonin and ATP in both awake and anesthetized rats subjected to hypoxia and hypercapnia (Fuller et al. 2000; Kinkead and Mitchell 1999; Kinkead et al. 2001).

The results in this study that show there is little or no glutamatergic signaling involved in the control of CVNs post
H/H is surprising. NMDA and non-NMDA receptor antagonists did not significantly inhibit the spontaneous and inspiratory-related EPSCs in CVNs post H/H. However, this study reveals the first endogenously active role of serotonergic neurotransmission to CVNs. Within the nucleus ambiguous CVNs receive the most dense axo-somatic 5-HT contacts in the brain stem (Takeuchi et al. 1983). However, the physiological role of the 5-HT contacts has not been previously elucidated. This study establishes a critical role for 5-HT3 receptors as a 5-HT pathway to CVNs is selectively recruited post H/H. The 5-HT3 receptor antagonist ondansetron inhibited both the spontaneous and inspiratory-related excitation of CVNs during recovery from hypoxia and hypercapnia, indicating both spontaneous and inspiratory-evoked 5-HT activity to CVNs are increased post H/H. The present work also demonstrates P2X receptors are involved and partly mediate an additional inspiratory-evoked selective purinergic excitation of CVNs post H/H.

In conclusion, normal brain stem cardiorespiratory interactions and the central cardiorespiratory responses to H/H are complex. Under control conditions, RSA is mediated by increases in GABA and glycine activity during inspiratory activity with no changes in excitatory glutamatergic, serotonergic, or purinergic neurotransmission to CVNs. The initial responses to H/H include an increase in inhibitory GABA and glycine neurotransmission to CVNs that is likely responsible for the initial tachycardia that occurs with H/H. This first phase is replaced by reduced GABA and glycine neurotransmission, and this likely is responsible for the bradycardia that follows the tachycardia in the biphasic responses to H/H. Finally there is a phase of augmented parasympathetic activity in the recovery period post H/H (Pichot et al. 2000; Roche et al. 2002). This maintained increase in parasympathetic cardiac vagal activity post H/H is not mediated by glutamatergic neurotransmission but is rather mediated by the recruitment of an excitatory purinergic pathway to CVNs evoked during inspiratory activity as well as the recruitment of a serotonergic pathway that elicits both spontaneous and inspiratory-related 5-HT3-mediated EPSCs in CVNs.

GRANTS

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REFERENCES


