Purinergic P2X Receptors Mediate Excitatory Transmission to Cardiac Vagal Neurons in the Nucleus Ambiguus After Hypoxia

Kathleen J. Griffioen, Christopher Gorini, Heather Jameson, David Mendelowitz

Abstract—Challenges such as hypoxia elicit a powerful response from both the central cardiovascular and respiratory neuronal networks. Recent work indicates that purinergic neurotransmission in the brain stem is an important modulator of central respiratory network responses to hypoxia. This study tests whether alterations in purinergic neurotransmission extend beyond respiratory responses to hypoxia and also mediates respiratory inputs to cardiac vagal neurons. To examine central cardiorespiratory responses to hypoxia, we used an in vitro medullary slice that allows simultaneous examination of rhythmic respiratory-related activity and synaptic neurotransmission to cardioinhibitory vagal neurons. Here we show that P2X receptor activation mediates respiratory-related excitatory neurotransmission to parasympathetic cardiac vagal neurons, the dominant control of heart rate. These data demonstrate a critical functional role for adenosine 5′-triphosphate–mediated purinergic signaling in facilitating respiratory-related excitatory neurotransmission to cardiac vagal neurons after hypoxia. (Hypertension. 2007;50:75-81.)

Key Words: nucleus ambiguus ■ parasympathetic ■ hypoxia ■ purinergic ■ cardiorespiratory

Heart rate is principally controlled by the activity of parasympathetic cardioinhibitory vagal neurons (CVN) located within the nucleus ambiguus. The activity of CVNs is intricately linked to respiratory function, and respiratory-related inputs to CVNs are critical for coordinated homeostatic interactions. For example, respiratory sinus arrhythmia, in which heart rate increases during each inspiration to optimize blood flow to the lungs, is mediated by increases in both inhibitory GABAergic and glycnergic neurotransmission to CVNs during inspiration.

Although respiratory sinus arrhythmia benefits pulmonary gas exchange by improving ventilation:perfusion ratios, respiratory dysfunction presents a major challenge to the cardiorespiratory system. Hypoxia transforms eupnea to gasping and elicits a parasympathetically mediated bradycardia, which decreases metabolic demands on the heart and increases survival. Although it is known that respiratory neurons in the brain stem directly respond to hypoxia, the cellular mechanisms by which central hypoxia alters the activity of parasympathetic cardiac vagal neurons has only recently been examined.

The purine nucleotide ATP is a clearly identified neurotransmitter within the central nervous system. ATP is released synaptically to mediate both presynaptic and postsynaptic effects at inotropic P2X and/or metabotropic P2Y cell surface receptors. Purinergic signaling is an important component of central respiratory network responses to hypoxia. ATP is released in the ventral medulla during hypoxia, and purinergic receptor antagonists alter the hypoxic respiratory response. Although P2 receptors are present within the medulla and contribute to respiratory control, very little is known regarding the purinergic contribution to respiratory modulation of parasympathetic cardiac neurons or responses to hypoxia in brain stem parasympathetic cardiac neurons.

We have shown previously that intermittent hypoxia recruits an excitatory neurotransmission to CVNs dependent on the generation of reactive oxygen species. However, the mechanisms regulating respiratory-related glutamatergic neurotransmission to CVNs in response to continuous hypoxia are unknown. Therefore, this study was designed to delineate the mechanisms mediating increases in respiratory-evoked glutamatergic neurotransmission on recovery from hypoxia. Here we show that activation of ATP receptors mediates hypoxia-induced recruitment of respiratory-related glutamatergic neurotransmission to CVNs. Specifically, we show that inotropic P2X receptors activate glutamatergic neurotransmission to CVNs on recovery from hypoxia.

Methods

Animal procedures are published as an online supplement available at http://hyper.ahajournals.org and are identical to previously published methodologies. All of the animal procedures were performed with the approval of the George Washington University.
Animal Care and Use Committee in accordance with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Recording Respiratory Network Activity
The thick medullary slice generates rhythmic respiratory-related motor discharge in hypoglossal cranial nerves. Spontaneous respiratory-related activity was recorded by monitoring motoneuron population activity from hypoglossal nerve rootlets using a suction electrode. Hypoglossal rootlet activity was amplified 50 000 times, filtered (10 to 300 Hz bandpass; CWE), and electronically integrated (τ=50 ms; CWE).

Patch-Clamp Techniques
CVNs in the nucleus ambiguus were identified by the presence of the fluorescent tracer, as described previously.11 Slices were viewed with infrared illumination and differential interference optics (Zeiss) and under fluorescent illumination with an infrared-sensitive cooled charged-coupled device camera (Photometrics). Neurons containing fluorescent tracer were identified by superimposing the fluorescent and infrared images on a video monitor (Sony).

Continuous focal drug application was performed using a pneumatic picopump pressure delivery system (WPI). Drugs were continuously ejected from a patch pipette positioned within 30 μm downstream from the drug pipette and considerably less behind the drug pipette.12 Glutamatergic neurotransmission was isolated by continuous focal application of strychnine hydrochloride (1 μmol/L) and gabazine (25 μmol/L) to block glycine and γ-aminobutyric acid receptors, respectively. In some experiments, suramin (100 μmol/L) or pyridoxal-phosphate-6-azophenyl-2,4′-disulfonic acid (PPADS; 100 μmol/L) to block P2 purinergic receptors or 2′(3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP; 100 μmol/L) to block purinergic P2X receptors was included in the focal drug application pipette. All of the drugs were obtained from Sigma.

Hypoxia
Rhythmic inspiratory-related activity and glutamatergic excitatory postsynaptic currents (EPSC) in a single CVN were recorded simultaneously for 6 minutes in control artificial cerebrospinal fluid (125 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L CaCl2, 26 mmol/L NaHCO3, 5 mmol/L glucose, and 5 mmol/L HEPES, equilibrated with 95% O2 and 5% CO2; pH 7.35 to 7.4). Hypoxia was induced by changing the control perfusate to an identical solution bubbled with 125 mmol/L NaCl, 3 mmol/L KCl, 26 mmol/L HEPES, 1 mmol/L of CaCl2, and 1 mmol/L of MgCl2 at a pH of 7.35 to 7.4. Voltage clamp recordings were made with Axopatch 200B and pClamp 8 software (Axon Instruments). All of the synaptic activity in CVNs was recorded at −80 mV. Only 1 experiment was performed per preparation.

As reported previously, there is no significant change in glutamatergic neurotransmission to CVNs during respiratory bursts under control or hypoxic conditions.4 However, on recovery from hypoxia, there is a significant increase in glutamatergic neurotransmission to CVNs simultaneous with respiratory bursts (see Figure 1; n=8; P<0.05).

Effect of Tempol on Central Cardiorespiratory Responses to Hypoxia
Reactive oxygen species are strongly implicated in physiological adaptations after hypoxia.13–15 In addition, we have shown recently that intermittent hypoxia recruits a respiratory-related glutamatergic neurotransmission to CVNs, dependent on the generation of reactive oxygen species.16 To test whether the enhancement of glutamatergic neurotransmission to CVNs on recovery from hypoxia is also mediated by reactive oxygen species generation, we used the superoxide dismutase mimetic Tempol (1 mmol/L). Tempol efficiently catalyzes the dismutation of free radicals.17–19 Application of Tempol did not significantly alter the increase in excitatory neurotransmission to CVNs on recovery from hypoxia (see Figure 1; n=8; P>0.05).

Effect of Purinergic Antagonists on Central Cardiorespiratory Responses to Hypoxia
ATP has been identified recently as an important mediator of central chemosensory and respiratory responses to hypoxia. To test whether activation of purinergic receptors by ATP also mediates the changes in excitatory neurotransmission to CVNs on recovery from hypoxia, we used the relatively broad P2 receptor antagonist PPADS20 to block both P2X and P2Y receptors (100 μmol/L). Focal application of PPADS did not alter glutamatergic neurotransmission to CVNs under control conditions or during hypoxia. However, on recovery from hypoxia, PPADS blocked the increase in excitatory neurotransmission to CVNs during respiratory bursts (see Figure 2; n=8; P<0.05). Focal application of the broad P2 receptor antagonist suramin had similar effects (n=4; P<0.05).

To further characterize the purinergic receptors responsible for recruiting an excitatory pathway to CVNs during inspiratory bursts on recovery from hypoxia, the more selective P2X receptor antagonist TNP-ATP21,22 (100 μmol/L) was used. Focal application of TNP-ATP also blocked the significant enhancement of glutamatergic neurotransmission to CVNs during recovery from hypoxia (see Figure 2, bottom; n=7).

Although TNP-ATP is often used to specifically examine P2X receptors, it may have broader actions. To further
characterize the role of P2X and P2Y receptors in facilitating glutamatergic neurotransmission to CVNs, we focally applied \( \alpha,\beta \)-methylene ATP (100 \( \mu \)mol/L), a selective agonist for P2X receptors. \( \alpha,\beta \)-Methylene ATP significantly increased glutamatergic EPSC frequency in CVNs (see Figure 3). Application of TNP-ATP (100 \( \mu \)mol/L) blocked the increase in glutamatergic EPSC frequency evoked by \( \alpha,\beta \)-methylene ATP (Figure 3). Furthermore, we also focally applied the P2Y agonists UTP (15 \( \mu \)mol/L) and adenosine 5'-0-(Z-thiodiphosphate) (60 \( \mu \)mol/L) to activate P2Y receptors that link to Gs and could, therefore, facilitate glutamatergic neurotransmission to CVNs. Application of UTP and adenosine 5'-0-(Z-thiodiphosphate) did not significantly increase glutamatergic frequency (control: 2.5±0.2 Hz; UTP and adenosine 5'-0-(Z-thiodiphosphate): 2.7±0.3 Hz; \( n = 6 \)), suggesting that P2X but not P2Y receptors enhance glutamatergic neurotransmission to CVNs.

Figure 1. Central cardiorespiratory responses to hypoxia are not mediated by reactive oxygen species generation. A, Inspiratory bursting activity was recorded from the hypoglossal rootlet (middle) and integrated (top). Fluorescently identified CVNs were patch clamped in the whole-cell configuration, and glutamatergic neurotransmission was isolated by focally application of gabazine (25 \( \mu \)mol/L) and strychnine (1 \( \mu \)mol/L). B, Average data from 8 cells, 3 seconds before and 3 seconds after burst. CVNs did not receive inspiratory-related modulation of excitatory neurotransmission before or during hypoxia \( P > 0.05 \). However, during recovery from hypoxia, CVNs received significant increases in glutamatergic neurotransmission simultaneous with inspiratory bursts \( P < 0.01 \). C, Continuous focal application of the superoxide dismutase mimetic tempol did not alter increases in glutamatergic neurotransmission to CVNs on recovery from hypoxia \( n = 8 \); \( P < 0.05 \). Asterisks denote that the frequency during the inspiratory burst is significantly greater than noninspiratory periods (ANOVA with repeated measures, \( P < 0.05 \)). Bar 0 represents the average frequency during inspiratory bursts, and error bars represent SEM.
Discussion

Purinergic receptor activation is a critical component of central cardiovascular and respiratory regulation. Purinergic receptors are present in key central cardiovascular and respiratory control centers, such as the nucleus of the solitary tract, rostroventrolateral medulla, ventral respiratory group, and hypoglossal nucleus. The extracellular ATP concentration in the brain stem rises in response to hypoxia and hypercapnia and mediates chemosensory transduction. Furthermore, activation of purinoceptors helps shape the central respiratory network response to hypoxia; ATP receptor activation helps maintain gasping and prevents excessive respiratory slowing. Our study extends the role of purinergic signaling to the central cardiorespiratory network; ATP receptor activation is essential for the respiratory-related excitatory synaptic neurotransmission to parasympathetic CVNs that control heart rate. In the recovery period after hypoxia, when heart rate is reduced, cardioinhibitory parasympathetic CVNs receive significant increases in glutamatergic neurotransmission during respiratory bursts, and the acti-

Figure 2. P2 purinergic receptors mediate glutamatergic neurotransmission to CVNs during recovery from hypoxia. A, Preparation and abbreviations are same as in Figure 1. Continuous focal application of the nonselective P2 receptor antagonist PPADS did not alter excitatory neurotransmission under control or hypoxic conditions. However, during recovery, PPADS blocked the respiratory-related glutamatergic neurotransmission to CVNs, as shown in a typical example. B, Average data from 8 cells, 3 seconds before and 3 seconds after burst. Continuous focal application of the selective P2X receptor antagonist TNP-ATP did not alter excitatory neurotransmission to CVNs under control or hypoxic conditions. C, However, during recovery, TNP-ATP also blocked respiratory-related glutamatergic neurotransmission to CVNs (average data from 7 cells, 3 seconds before and 3 seconds after burst). Asterisks denote that the frequency during the inspiratory burst is significantly greater than noninspiratory periods (ANOVA with repeated measures, \( P < 0.05 \)). Bar 0 represents the average frequency during inspiratory bursts, and error bars represent SEM.
vation of this pathway depends on activation of P2X receptors.

We have shown recently that reactive oxygen species are required for the recruitment of an excitatory neurotransmission to CVNs during acute intermittent hypoxia. It is, therefore, somewhat surprising that inhibition of reactive oxygen species generation with the nitroxide Tempol did not alter the enhanced glutamatergic neurotransmission on recovery from a single episode of hypoxia in this study. However, we have shown previously that reactive oxygen species are not significantly generated within the ventrolateral medulla either during or on recovery from a single episode of hypoxia, but reactive oxygen species are incrementally generated during intermittent periods of hypoxia. Reactive oxygen species generation is reported during recovery from hypoxia in the forebrain and nodose ganglion neurons. These data suggest that intermittent and sustained hypoxia evoke functionally distinct mechanisms of cardiorespiratory plasticity.

Several P2X receptor subtypes have been identified within the nucleus ambiguus. This is the first report identifying a functional role for P2X receptor activation in mediating excitatory neurotransmission to CVNs. P2X receptors are nonselective cation channels with equal permeability to potassium and sodium and a significant permeability to calcium. Activation of P2X receptors is reported to facilitate neurotransmitter release by direct calcium entry through P2X receptors or through activation of voltage-gated calcium channels. Therefore, P2X receptors may facilitate glutamate release by directly mediating presynaptic calcium entry or, alternatively, by depolarizing the presynaptic terminal to open voltage-gated calcium channels that then elicit glutamate release. The purinergic modulation of excitatory neurotransmission described in this study is similar to properties of purinergic modulation of glutamatergic neurotransmission that have been reported elsewhere within the central nervous system, including the hippocampus and nucleus of the solitary tract.

The nonselective P2 receptor antagonist PPADS inhibited respiratory-related increases in glutamatergic neurotransmission during recovery from hypoxia. Because PPADS was focally applied to cardiac vagal neurons, ATP is likely acting on P2 receptors in close proximity to CVNs to enhance the release of glutamate. Focal application of the selective P2X agonist α,β-methylene ATP but not the P2Y agonists UTP and adenosine 5'-O-(Z-thiodiphosphate) facilitated glutamatergic EPSCs in CVNs also suggesting P2X but not P2Y receptors are localized on glutamatergic synaptic terminals on CVNs and can enhance excitatory neurotransmission to CVNs.

However, whether P2 receptor activation at distant sites may also contribute to glutamatergic neurotransmission to cardiac vagal neurons is unknown. Purinergic neurotransmission is a critical component of chemosensory and respiratory responses to hypoxia and hypercapnia. In addition, during hypoxia, ATP is also released on the dorsal surface of the medulla, near the nucleus of the solitary tract, which activates an excitatory pathway to cardiac vagal neurons. Consistent with this, microinjection of P2 receptor agonists into the nucleus of the solitary tract induces bradycardia. Therefore, whereas local P2 receptor activation mediates enhanced excitatory neurotransmission to cardiac vagal neurons after hypoxia, purinergic neurotransmission in other functionally relevant cardiorespiratory control sites may also contribute to excitatory neurotransmission to cardiac vagal neurons.

The cellular origin of purinergic neurotransmission within the central nervous system remains unknown. ATP may be synaptically released from respiratory-related neurons that innervate cardiac vagal neurons. However, central neural origins of ATP neurotransmission are not well defined. Alternatively, ATP may originate from exocytotic vesicular release from astrocytes. ATP is released from cortical

![Figure 3](http://hyper.ahajournals.org/figure/3/)

Figure 3. Focal application of the selective agonist for P2X receptors, α,β-methylene ATP (100 μmol/L), significantly increased glutamatergic EPSC frequency in CVNs (top traces). Application of TNP-ATP (100 μmol/L) blocked the increase in glutamatergic EPSC frequency evoked by α,β-methylene ATP (middle). Results from typical experiments are shown in the 2 series of traces, and the average results from 7 CVNs are shown in the bar graph (bottom; *P<0.05).
astrocytes in response to NO,39 and within the spinal cord, ATP is released from astrocytes to mediate nociception.40 Alternatively, astrocytes can release glutamate through ATP-evoked vesicular release.31,42 Indeed, stimulation of P2X7 receptors on astrocytes is reported to evoke glutamate release.43 Further studies are required to determine the pathway(s) and source(s) of purinergic neurotransmission to CVNs.

Perspectives
Excitatory synaptic pathways to premotor cardiac vagal neurons are recruited with respiratory bursts in the recovery period from hypoxia. In adults, this excitatory response may be involved in the potentially life-threatening arrhythmias that occur with obstructive sleep apnea. Recovery from obstructive sleep apnea in humans either occurs by arousal from sleep or from hypoxic gasping (autoresuscitation) that is often accompanied by nocturnal and severe bradycardia and bradyarrhythmic.44,45 Atropine, a blocker of cardiac receptors, is reported to evoke glutamate release.46 Furthermore, our data are consistent with studies that report increases in parasympathetic outflow after apnea in healthy infants.47 Although the cause of bradycardia associated with apnea is unknown, this study suggests that these heart rate changes during recovery from apnea may be mediated by respiratory-related excitation of CVNs subsequent to P2X receptor activation.

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Disclosures
None.

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Animal Preparation

Sprague Dawley rat pups (postnatal day 1-3; Hilltop, Scottsdale, PA) were anesthetized and exposed to hypothermia to slow the heart. The heart was exposed by a right thoractomy and the retrograde fluorescent tracer X-rhodamine-5-(and 6)-isothiocyanate (Molecular Probes, Eugene, OR) was injected into the fat pads at the base of the heart. After 24-48 hours recovery, pups were anesthetized with halothane, sacrificed by cervical dislocation, and the hindbrain was rapidly removed and placed in cold physiological saline solution (140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl$_2$, 5 mmol/L glucose, 10 mmol/L HEPES, bubbled with 100% O$_2$, pH 7.4). The medulla was carefully removed, preserving the hypoglossal nerve rootlets. The medulla was mounted on a cutting block rostral side up and placed in a vibrating blade microtome (Leica, Nussloch, Germany). Serial coronal sections of the medulla were sliced until the inferior olive and the nucleus ambiguus were visualized on the rostral surface of the tissue. A single thick slice of the medulla (800 μm) was made that included CVNs, the rostral hypoglossal nucleus and rootlets, and the PreBotzinger complex. This slice was placed in a recording chamber that allowed perfusion (4 mL/min) with artificial cerebrospinal fluid (125 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L CaCl$_2$, 26 mmol/L NaHCO$_3$, 5 mM glucose, 5 mmol/L HEPES, equilibrated with 95% O$_2$, 5% CO$_2$, pH 7.35-7.4).