Multiple Types of GABA_\text{A} Receptors Mediate Inhibition in Brain Stem Parasympathetic Cardiac Neurons In the Nucleus Ambiguus

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Bouairi, Eugenia, Harriet Kamendi, Xin Wang, Christopher Gorini, and David Mendelowitz. Multiple types of GABA_\text{A} receptors mediate inhibition in brain stem parasympathetic cardiac neurons. J Neurophysiol 96: 3266–3272, 2006. First published August 16, 2006; doi:10.1152/jn.00590.2006. Recent work suggests neurons can have different types of \(\gamma\)-aminobutyric acid type A (GABA_\text{A}) receptors that mediate phasic inhibitory postsynaptic currents (IPSCs) and tonic currents. This study examines the diversity of GABAergic synaptic currents in parasympathetic cardioinhibitory neurons that receive rhythmic bursts of GABAergic neurotransmission. Focal application of gabazine (25 \(\mu\)M) to cardiac vagal neurons in vitro did not change the frequency of firing in spontaneously active neurons or the resting membrane potential; however, picrotoxin (100 \(\mu\)M) significantly depolarized cardiac vagal neurons and increased their firing. Similarly, gabazine (25 \(\mu\)M) selectively blocked GABAergic IPSCs but did not change holding current in cardiac vagal neurons, whereas picrotoxin (100 \(\mu\)M) not only blocked GABAergic IPSCs but also rapidly decreased the tonic current. Because the tonic current could be attributable to activation of GABA receptors by ambient GABA or, alternatively, spontaneous opening of constitutively active GABA channels, an antagonist for the GAT-1 GABA transporter NO-711 (10 \(\mu\)M) was applied to distinguish between these possibilities. NO-711 did not significantly alter the holding current in these neurons. The benzodiazepine flunitrazepam (1 \(\mu\)M) significantly increased the tonic current and GABAergic IPSC decay time; surprisingly, however, in the presence of gabazine flunitrazepam failed to elicit any change. These results suggest cardiac vagal neurons possess gabazine-sensitive GABA_\text{A} receptors that mediate phasic synaptic currents, a gabazine-insensitive but picrotoxin-sensitive extrasynaptic tonic current that when blocked depolarizes and increases the firing rate of cardiac vagal neurons, and benzodiazepines recruit a third type of GABA_\text{A} receptor that is sensitive to gabazine and augments the extrasynaptic tonic current.

INTRODUCTION

\(\gamma\)-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian CNS, including the brain stem. Synaptically released GABA activates postsynaptic GABA_\text{A} receptors, which increase the membrane permeability to chloride, evoking a hyperpolarizing inhibitory postsynaptic current (IPSC). Synaptic release increases the concentration of GABA to a relatively high level (millimolar range) within the synapse (Farrant and Nusser 2005). The brief current evoked by synaptic release of GABA from presynaptic terminals is referred to as “phasic” inhibition.

However, recent work has indicated GABA released from presynaptic terminals can escape, or spill over, from the synaptic cleft and activate receptors either at, or distant from, the synapse (Farrant and Nusser 2005). The current elicited from this leakage of GABA from the synapse is thought to cause a persistent or “tonic” activation of GABA_\text{A} receptors, resulting in a maintained hyperpolarizing current. Recent work has suggested the GABA_\text{A} receptors responsible for the phasic and tonic currents have different pharmacological properties, which can be different depending on the cell type (Farrant and Nusser 2005). Phasic currents can be selectively blocked by gabazine (SR-95531), which blocks the binding of GABA to its binding site in the receptor, whereas the pore blocker picrotoxin blocks both the phasic and tonic currents (Semyanov et al. 2003). It has also been proposed that benzodiazepines differentially alter GABA_\text{A} receptors, prolonging the decay time of phasic IPSCs, without altering the tonic conductance evoked by extrasynaptic GABA_\text{A} receptors (Nusser and Mody 2002).

The importance of phasic inhibition by activation of GABA_\text{A} receptors in premotor parasympathetic cardioinhibitory neurons in the brain stem is well documented. Stimulation of sensory afferents evokes a biphasic pattern consisting of an initial excitation followed by a GABAergic-mediated inhibition of parasympathetic cardioinhibitory neurons (Evans et al. 2003). The inhibitory neurotransmission likely acts to increase entrainment of cardioinhibitory parasympathetic activity to the increase in blood pressure that occurs with each heartbeat (Evans et al. 2003). Respiratory sinus arrhythmia, in which the heart rate slows during each inspiration, is mediated by interactions between the respiratory and cardiovascular systems in the brain stem and has been shown in adult animals to be mediated by an inhibition during inspiration, which can be reversed by the intracellular injection of Cl\(^-\) (Gilbey et al. 1984), and is mediated in postnatal animals by bursts of GABAergic neurotransmission to parasympathetic cardioinhibitory neurons that occurs with each inspiration (Neff et al. 2003, 2004). Respiratory sinus arrhythmia helps match pulmonary blood flow to lung inflation and maintain an appropriate diffusion gradient for oxygen in the lungs (Taylor et al. 1999).

However, little is known about the presence or role of tonic activation of GABA_\text{A} receptors in these brain stem neurons. The role of a tonic inhibition may play an especially important role in parasympathetic cardioinhibitory neurons because cardiac vagal neurons receive rhythmic respiratory-related high-frequency bursts of GABAergic neurotransmission. This study tests whether cardiac vagal neurons possess a tonic GABAergic current and examines the pharmacological properties and...
differences of the phasic and tonic currents in these neurons. The results in this study indicate there are multiple types of GABA\textsubscript{A} receptors that are involved in both tonic and phasic GAB\textsubscript{A}ergic inhibition of brain stem parasympathetic cardioinhibitory neurons.

**Methods**

Sprague–Dawley rat pups (postnatal days 1–7; Hilltop, Scottsdale, PA) were anesthetized with ketamine–xylazine (87/13 mg/kg) 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 a 24- to 48-h recovery, pups were anesthetized with halothane, killed by cervical dislocation, and the hindbrain was rapidly removed and placed in cold physiological saline solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl\textsubscript{2}, 5 mM glucose, 10 mM HEPES, bubbled with 100% O\textsubscript{2}, pH 7.4).

For immunohistochemical experiments the medulla was mounted on a cutting block and placed in a vibrating blade microtome (Leica, Nussloch, Germany). Sections of the brain stem (about 900 \( \mu \)m thickness) that contain the nucleus ambiguus were fixed overnight at 4°C in 75.7% picric acid and 0.81% paraformaldehyde in phosphate buffer. Slices were cryoprotected at 4°C in 30% sucrose solution for about 2 h. The tissue was then embedded in OCT embedding medium (Sakura Finetek, Torrance, CA) at −20°C and sectioned into 40- to 50-\( \mu \)m-thick slices using a cryostat. Slices were then washed three times in 0.1 M phosphate-buffered saline (PBS) followed by 1 h incubation with 5% normal donkey serum (Jackson Antibodies, Rockville, MD) and 0.04% Triton-X in 0.1 M PBS. The tissue was immersed in a primary antibody mixture containing 1:100 dilution of goat anti-choline acetyltransferase (ChAT, Chemicon, Temecula, CA), 5% normal donkey serum and 0.04% Triton-X in 0.1 M PBS overnight at 4°C. By 12 h later the slices were rinsed three times at room temperature and incubated in a 1:200 dilution of Alexa Fluor 647 donkey anti-goat IgG secondary antibody (Invitrogen, Cambridget, MA) in 0.1 M PBS for 2 h at room temperature, followed by three washes. The tissue was then mounted on glass slides, air dried, and then overslipped with hard-set antifading mounting media (Vector Laboratories, Burlingame, CA). Control tissue adjacent slices were used and the ChAT antibody was substituted with normal goat serum, in a dilution comparable to that used for the primary antibody. The immunofluorescence was visualized with an Axiovert 200 microscope fitted with an AxioCam MRm digital camera and AxioVision software (Carl Zeiss, Oberkochen, Germany).

For electrophysiological experiments slices of the medulla (300–500 \( \mu \)m) were made that included parasympathetic cardioinhibitory neurons and the tissue was placed in a recording chamber that allowed perfusion (4 mL/min) with artificial cerebrospinal fluid (125 mM NaCl, 3 mM KCl, 2 mM CaCl\textsubscript{2}, 26 mM NaHCO\textsubscript{3}, 5 mM glucose, 5 mM HEPES, equilibrated with 95% O\textsubscript{2},5% CO\textsubscript{2}, pH 7.35–7.4). 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 Guide for the Care and Use of Laboratory Animals.

Parasympathetic cardioinhibitory neurons in the nucleus ambiguus were identified by the presence of the fluorescent tracer, as described previously (Mendelowitz 1996). Specificity of the cardiac vagal labeling is confirmed by the absence of any labeled neurons in the brain stem when rhodamine is injected into the chest cavity while keeping the pericardial sac intact, or when the injection into the pericardial sac is accompanied by section of the cardiac branch of the vagus nerve \((n = 4)\). In other control experiments \((n = 10)\) intravenous injection of \( \leq 10 \) mg of rhodamine failed to label any neurons in the medulla except for rare labeling of neurons in the area postrema, an area with a deficient blood–brain barrier. 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, Tucson, AZ). Neurons containing fluorescent tracer were identified by superimposing the fluorescent and infrared images on a video monitor (Sony, Tokyo, Japan).

Patch pipettes (2.5–4.5 M\( \Omega \)) were visually guided to the surface of individual CVNs using differential interference optics and infrared illumination (Zeiss). In voltage-clamp experiments pipettes were filled with a solution containing 150 mM KCl, 4 mM MgCl\textsubscript{2}, 2 mM EGTA, 2 mM Na-ATP, and 10 mM HEPES, pH 7.4. This pipette solution causes the Cl\textsuperscript{−} current induced by the activation of GABA\textsubscript{A} receptors to be recorded as an inward current at a holding voltage of −80 mV. In current-clamp experiments the pipette solution was 130 mM K-gluconate, 10 mM HEPES, 10 mM EGTA, 1 mM CaCl\textsubscript{2}, and 1 mM MgCl\textsubscript{2}. Voltage- and current-clamp recordings were made with Axopatch 200B and pClamp 8 software (Axon Instruments, Union City, CA). All synaptic activity in parasympathetic cardioinhibitory neurons was recorded at −80 mV. No more than one experiment was performed in each slice of tissue.

Focal drug application was performed using a pneumatic picopump pressure delivery system (WPI, Sarasota, FL). Drugs were ejected from a patch pipette positioned within 30 \( \mu \)m of the patched CVN. The maximum range of drug application was previously determined to be 100 to 120 \( \mu \)m downstream from the drug pipette and considerably less behind the drug pipette (Wang et al. 2002). GABA\textsubscript{A}ergic neurotransmission was isolated by inclusion in the perfusate of d-2-amino-5-phosphonovalerate (AP5, 50 \( \mu \)M), 6-cyano-7-nitroquinoline-2,3-dione (CNQX, 50 \( \mu \)M), and strychnine hydrochloride (1 \( \mu \)M) to block N-methyl-D-aspartate (NMDA), non-NMDA, and glycine receptors, respectively. All drugs were obtained from Sigma.

Analysis of spontaneous synaptic currents was performed using MiniAnalysis (version 5.6.12, Synaptosoft) with minimal acceptable amplitude at 6–9 pA. Results are presented as means ± SE. Statistical comparisons were performed using a paired t-test and significant difference was set at \( P < 0.05 \).

**Results**

All cardiac vagal neurons identified with the retrograde fluorescent tracer rhodamine expressed choline acetyltransferase (ChAT), as expected (see Fig. 1). However cardiac vagal neurons constituted only a small proportion (roughly 15–20%) of the population of ChAT positive neurons in the nucleus ambiguus, with noncardiac ChAT positive neurons located in the compacta formation. Cardiac vagal neurons were typically located at the ventral perimeter of the nucleus ambiguus, referred to as the external formation, and were generally smaller than the ChAT positive neurons in the compacta formation of the nucleus ambiguus (Fig. 1).

Application of gabazine (25 \( \mu \)M) did not change the resting membrane potential in cardiac vagal neurons recorded in current-clamp configuration (see Fig. 2). In contrast, focal application of picrotoxin (100 \( \mu \)M) significantly \((P < 0.01)\) depolarized cardiac vagal neurons. Nearly all cardiac vagal neurons were silent and devoid of spontaneous discharge, consistent with previous reports (Mendelowitz 1996); however, one cardiac vagal neuron (out of 17) did have a low level of spontaneous firing (0.033 Hz) (see Fig. 2, bottom). Application of gabazine (25 \( \mu \)M) did not induce any silent cardiac vagal neurons to fire and did not change the firing frequency in the one spontaneously firing cardiac vagal neuron. However, application of picrotoxin (100 \( \mu \)M) increased the firing rate in the...
spontaneously active cardiac vagal neuron (from 0.033 to 0.193 Hz) and also evoked a depolarization sufficient to elicit firing in three additional cardiac vagal neurons (average frequency of 0.22 Hz).

To characterize the synaptic gabazine-sensitive and extrasynaptic gabazine-insensitive but picrotoxin-sensitive GABAergic responses in more detail the spontaneous GABAergic currents were examined in voltage-clamp configuration. Gabazine (25 μM) selectively blocked the GABAergic inhibitory postsynaptic currents (IPSCs), but did not significantly change the holding current in cardiac vagal neurons (control: −81 ± 12 pA, gabazine: −79 ± 13 pA, n = 16) (see Fig. 3, top). In contrast, picrotoxin (100 μM) not only blocked the GABAergic IPSCs, but also rapidly and significantly decreased the tonic holding current from −95 ± 17 to −50 ± 12 pA (P < 0.01, n = 16).

Two different mechanisms responsible for the tonic picrotoxin-sensitive and gabazine-insensitive current could be activation of GABA receptors by ambient GABA or, alternatively, spontaneous opening of constitutively active GABA channels. If ambient GABA is responsible it is likely the tonic current would be modulated by agents that increase the extracellular concentration of GABA, whereas spontaneous opening of constitutively active GABA channels would be unaffected. To help distinguish between these possibilities an antagonist for the GAT-1 GABA transporter, NO-711 (10 μM), was included in the perfusate. NO-711 did not significantly alter the holding current in these neurons, but, similar to other reports in which the GABA transporter inhibitor NO-711 prolongs the decay time of IPSCs (Keros and Hablitz 2005; Petrini et al. 2004; Semyanov et al. 2003; Wei et al. 2003), inhibition of the GAT-1 transporter in the brain stem significantly increased the decay time of the IPSCs from 29 ± 1 to 32 ± 1 ms (P < 0.01, n = 10) in cardiac vagal neurons (see Fig. 4).

Because benzodiazepines can potentially distinguish between GABA<sub>A</sub> receptors consisting of different subunit combinations, the benzodiazepine flunitrazepam was focally applied to cardiac vagal neurons. Flunitrazepam (1 μM) significantly (P < 0.01) increased the tonic holding current (from −102 ± 24 to −147 ± 26 pA) and GABAergic IPSC decay time (from 42 ± 3 to 62 ± 2 ms) (see Fig. 5). Surprisingly, however, the flunitrazepam facilitation of the GABA receptors...
The increase in tonic holding current was gabazine sensitive because, in the presence of gabazine, flunitrazepam failed to elicit any change in tonic holding current (Fig. 6).

**DISCUSSION**

There are three major conclusions from this study. 1) Cardiac vagal neurons possess both gabazine-sensitive GABA\(_A\) receptors that mediate phasic synaptic currents and a gabazine-insensitive but picrotoxin-sensitive extrasynaptic tonic current that, when blocked, depolarizes and can evoke firing in cardiac vagal neurons. 2) Because the GABA transporter inhibitor NO-711 prolonged the decay time of IPSCs, but did not enhance the tonic current, it seems most likely that either a) the increase in ambient GABA with NO-711 is largely limited to the synapses surrounding parasympathetic cardiac neurons with little activation of extrasynaptic receptors, b) the normal ambient concentration of GABA within the brain stem is already sufficient to saturate the extrasynaptic receptors, or c) the picrotoxin-sensitive extrasynaptic tonic current is mediated by spontaneous opening of constitutively active GABA channels. 3) Benzodiazepines recruit a third type of GABA\(_A\) receptor that is sensitive to gabazine and, when facilitated by benzodiazepines, not only augments the extrasynaptic tonic current but also increases the decay time of phasic IPSCs.

Tonic inhibition by GABA\(_A\) receptors was previously shown in many types of neurons, such as hippocampal CA1 interneu-
rons, but not in others, such as pyramidal cells (Semyanov et al. 2003). In cerebellar and dentate granule cells the tonic GABA<sub>A</sub> receptor mediated current is thought be mediated by the high-affinity δ subunit containing GABA<sub>A</sub> receptors (Brickley et al. 2001; Nusser et al. 1998). In support of the role of δ subunit–containing GABA<sub>A</sub> receptors the δ subunit was earlier shown to be localized exclusively to extrasynaptic or perisynaptic sites (Nusser et al. 1998; Wei et al. 2003). Interestingly, it was proposed that the lack of γ subunit in these δ subunit–containing GABA<sub>A</sub> receptors is responsible for the lack of incorporation at the synapse (Farrant and Nusser 2005). However, the α5 subunit–containing GABA<sub>A</sub> receptors in hippocampal pyramidal neurons were also shown to be localized predominantly, if not exclusively, to extrasynaptic or perisynaptic sites and may also mediate a tonic inhibition (Farrant and Nusser 2005).

The mechanisms responsible for the activation of tonic current are unknown, but among the proposed possibilities include spillover of transmitter released from neighboring synapses, reverse operation of GABA transporters, release of GABA from astrocytes, and spontaneous opening of constitutively active GABA channels (Bai et al. 2001). Because the GAT-1 GABA transporter NO-711 (10 μM) did not significantly alter the tonic current in cardiac vagal neurons it is possible that the tonic current in these neurons is caused by spontaneous opening of constitutively active GABA channels, such as GABA channels that contain the ε subunit (Wagner et al. 2005). Alternatively, the tonic current may be caused by ambient GABA, such as from spillover of transmitter released from neighboring synapses if the ambient concentration of GABA is already saturating such that GABA transporters can evoke no additional response. This seems especially likely if the GABA channels responsible for the tonic current in cardiac vagal neurons contain δ receptors, which possess a very high affinity for GABA (Saxena and Macdonald 1996).

Cardiac vagal neurons are highly sensitive to benzodiazepines, which augment the extrasynaptic tonic current as well as increase the decay time of phasic IPSCs. Because the presence of a γ subunit is necessary for benzodiazepine modulation of GABA receptors (Gunther et al. 1995; Pritchett et al. 1989; Sigel et al. 1990), it is highly likely that at least some GABA<sub>A</sub> receptors in cardiac vagal neurons possess a γ subunit. The α subunit that likely accompanies the γ subunit containing GABA<sub>A</sub> receptors in cardiac vagal neurons is unknown. The α1 subunit is photoaffinity labeled by flunitrazepam, suggesting the binding site for benzodiazepines is likely

![FIG. 5. Benzodiazepine flunitrazepam (1 μM) significantly increased the tonic holding current and GABAergic IPSC decay time, as shown in a typical experiment (top and middle), with the average results (n = 9) shown at the bottom.](http://jn.physiology.org/doi/abs/10.1152/jn.00327.2006)
between the α1 and γ subunits (Sigel and Buhr 1997). It was also previously proposed that the presence of α1–α3 or α5 subunits is required for sensitivity to benzodiazepines (Luscher and Keller 2004). Although possible, it seems unlikely that either α2 or α5 subunits that contain GABA_A receptors are responsible because localization of the different α subunits indicates the α1 and α3 subunit–containing GABA_A receptors are densely expressed, whereas α2, α4, α5, and α6 subunits are relatively weakly expressed in the brain stem (Fritschy and Brunig 2003). Furthermore, α2 and α3 subunits are expressed mainly in the synapse, whereas α1, α4, α5, and α6 subunits are localized at both synaptic and extrasynaptic sites (Fritschy and Brunig 2003). It therefore seems most likely that the benzodiazepine-sensitive GABA_A receptors in cardiac vagal neurons, which augment the extrasynaptic tonic current as well as increase the decay time of phasic IPSCs, most likely contain α1 and γ subunits.

Although it is often assumed that the δ subunits containing GABA_A receptors are insensitive to benzodiazepines, which would thus rule out the role of δ subunit–containing GABA_A receptors in the benzodiazepine-facilitated tonic current in cardiac vagal neurons, there is little direct evidence for the conclusion that δ subunit–containing GABA_A receptors are insensitive to benzodiazepines and, in fact, some studies suggest the opposite. Diazepam did not increase GABA-evoked currents in α1β1δ GABA receptors, consistent with the hypothesis that the presence of a γ subunit is necessary for benzodiazepine modulation of GABA receptors, but the responses from α1β1γ2Lδ subunit–containing GABA receptors were sensitive to benzodiazepines and enhanced by diazepam (Saxena and Macdonald 1994). More recently it was shown that recombinant GABA receptors consisting of α6 and δ subunits are modulated by benzodiazepines (Santhakumar et al. 2006).

In summary, cardiac vagal neurons possess both gabazine-sensitive GABA_A receptors that mediate phasic synaptic currents and a gabazine-insensitive but picrotoxin-sensitive extrasynaptic tonic current that, when blocked, depolarizes and can evoke firing in cardiac vagal neurons. The functional role of the tonic GABAergic current is to likely set the membrane potential close to the equilibrium potential for GABA-induced chloride currents as well as increase membrane conductance, allowing changes in ambient GABA concentrations to directly change membrane potential and responsiveness to other synaptic inputs (Farrant and Nusser 2005). Activation of a tonic current in cardiac vagal neurons would decrease the neuron’s input resistance and effectively “shunt” excitatory inputs, further ensuring inhibition during bursts of GABAergic neurotransmission to cardiac vagal neurons. Because the GABA transporter inhibitor NO-711 prolonged the decay time of IPSCs, but did not enhance the tonic current, it seems most likely that either the tonic current is caused by spontaneous opening of constitutively active GABA channels, such as GABA channels that contain the ε subunit, or the ambient concentration of GABA in this area of the brain stem is already sufficiently high such that GABA transporter antagonists can evoke no additional response in already saturated GABA receptors. The benzodiazepine-sensitive responses in cardiac vagal neurons are possibly attributable to the recruitment of a third type of GABA_A receptor that is sensitive to gabazine and, when facilitated by benzodiazepines, augments the extrasynaptic tonic current and increases the decay time of phasic IPSCs.

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