Endogenous Acetylcholine and Nicotine Activation Enhances GABAergic and Glycinergetic Inputs to Cardiac Vagal Neurons

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INTRODUCTION

The neural control of heart rate and cardiac function are dominated by the parasympathetic activity to the heart, while cardiac sympathetic activity plays a minor role under resting conditions (Loewy and Spyer 1990; Mendelowitz 1999, 1996). Premotor cardiac vagal neurons are located in the nucleus ambiguus and the dorsal motor nucleus of the vagus (DiMicco et al. 1979; Mendelowitz 1999, 1996; Standish et al. 1995; Stuesse 1982). Since premotor cardiac vagal neurons are intrinsically silent, their activity is determined by excitatory and inhibitory synaptic inputs (Mendelowitz 1999, 1996; Mendelowitz and Kunze 1991).

Excitatory inputs to premotor cardiac vagal neurons include glutamatergic inputs from the nucleus tractus solitarius (NTS) and cholinergic nicotinic receptors that can directly excite premotor cardiac vagal neurons (Neff et al. 1998a; Wang et al. 2001a). In addition nicotine can enhance glutamatergic neurotransmission to premotor cardiac vagal neurons at both presynaptic and postsynaptic sites (Neff et al. 1998a; Wang et al. 2001a). The presynaptic facilitation can be blocked by antagonists specific for α7-subunit–containing nicotinic receptors and this presynaptic TTX-insensitive facilitation is dependent on the activation of voltage-gated calcium channels (Neff et al. 1998a; Wang et al. 2001a).

However considerably less is known about the function and modulation of inhibitory synaptic inputs to premotor cardiac vagal neurons. Stimulation of the NTS has been shown to evoke a monosynaptic inhibitory GABAergic pathway to premotor cardiac vagal neurons (Wang et al. 2001b). Although there have been no previous electrophysiological studies of glycinergetic innervation of premotor cardiac vagal neurons, these neurons also likely receive glycinergetic inputs, since microinjection of glycine into the nucleus ambiguus elicits tachycardia in spinal rats (Chitravanshi et al. 1991).

The inhibitory neurotransmission to premotor cardiac vagal neurons is likely involved in cardiorespiratory interactions. In each respiratory cycle, the heart beats more rapidly in inspiration and slows during postinspiration and expiration (referred to as respiratory sinus arrhythmia). In an in vivo study the input resistance decreased and premotor cardiac vagal neurons were hyperpolarized during inspiration, and the hyperpolarization was reversed upon injection of Cl− (Gilbey et al. 1984). Paradoxically, however, in a review it is stated the inspiratory-related inhibition of cardioinhibitory neurons was not antagonized by the iontophoretic application of either the GABA A antagonist bicuculline or the glycine antagonist strychnine (Loewy and Spyer 1990). More recent work in vitro has indicated premotor cardiac vagal neurons receive increased inhibitory GABAergic synaptic currents during inspiration and that this increased GABAergic activity during inspiration can be abolished by nicotinic receptor antagonists (Neff and Mendelowitz 2002).
The goals of the present study were 1) to test whether endogenous cholinergic activity modulates GABAergic and glycinegic neurotransmission to cardiac vagal neurons, 2) to test whether exogenous application of nicotine alters spontaneous GABAergic and glycinegic activity to premotor cardiac vagal neurons, and 3) to determine the nicotinic receptor subtypes involved and to investigate the sites of action of nicotine on the GABAergic and glycinegic neurons that synapse on premotor cardiac vagal neurons.

METHODS

In an initial surgery, 2- to 6-day-old rats were anesthetized with halothane and exposed to hypothermia during the surgery (10–20 min) to slow the heart and to aid in recovery. A right thoracotomy was used to expose the heart and rhodamine (XRRTC, 2% solution, 20–40 µl, Molecular Probes) was injected into the pericardial sac. Control injections of rhodamine either into the chest cavity but outside the pericardial sac or intravenously failed to label any neurons in the medulla, except for rare labeling of area postrema neurons observed with intravenous injections. On the day of the experiment (1–3 days later), the animals were anesthetized deeply with halothane and decapitated at the supracollicular level. The brain was submerged in cold (4°C) buffer of the following composition (mM): 140 NaCl, 5 KCl, 2 CaCl2, 5 glucose, and 10 HEPES, and continually gassed with 100% O2. Under a dissection microscope the cerebellum was removed and the hindbrain was isolated. The brain stem was then secured in the slicing chamber and submerged in cold saline containing: 125 NaCl, 3 KCl, 2 CaCl2, 5 glucose, and 10 HEPES, and continually bubbled with 100% O2. A right thoracotomy was performed using the whole-cell patch-clamp technique and were voltage clamped at a holding potential of −60 mV. The patch pipettes were filled with a solution consisting of (in mM) 150 KCl, 2 MgCl2, 2 EGTA, 10 HEPES, and 2 Mg-ATP, pH 7.35. With this patch pipette solution the CI− current induced by activation of GABA or glycine receptors was recorded as an inward current (calculated reversal potential of CI−: +4 mV).

To examine whether there is endogenous cholinergic modulation of either GABAergic or glycinegic synaptic activity to cardiac vagal neurons, neostigmine (10 µM), an acetylcholinesterase inhibitor, was applied by inclusion in the perfusate. 2D-Amino-5-phosphonovalerate (AP5, 50 µM) and 6-cyano-7-nitroquinolinolamine-2,3-dione (CNQX, 50 µM) were included in the perfusate to block glutamatergic receptors. GABAergic inhibitory postsynaptic currents (IPSCs) and mIPSCs were isolated by the inclusion of strychnine (1 µM) in the perfusate and glycinegic IPSCs and mIPSCs were isolated by the inclusion of either picrotoxin (1 µM) or gabazine (50 µM) in the perfusate. To determine whether activation of nicotinic receptors could mimic the cholinergic facilitation of GABAergic and glycinegic neurotransmission, nicotine (0.1–1 mM) was dissolved in perfusate and was locally applied for 10–20 s through a puffer pipette positioned within 10 µm of the neuron. To test whether this method of focal application produced any artifacts, the external solution was puffed onto cardiac vagal neurons with identical pressure settings and close proximity. Application of external solution had no significant effect on the frequency (1.4 ± 0.1, 1.2 ± 0.1 Hz, n = 4, P > 0.05), amplitude (174 ± 32, 171 ± 35 pA, n = 4, P > 0.05) of GABAergic IPSCs, or the holding current (−53 ± 11, −50 ± 14 pA, n = 4, P > 0.05). A similar lack of effect of puffing the perfusate occurred when glycinegic events were isolated (frequency, 1.3 ± 0.1, 1.3 ± 0.1 Hz, n = 4, P > 0.05; amplitude, 44 ± 2, 43 ± 1 pA, n = 4, P > 0.05; holding current, −53 ± 13, −52 ± 14 pA, n = 4, P > 0.05).

In some experiments nicotine was reapplied to a slice after a delay of 20 min to minimize any desensitization of the neurons in the slice. At the end of each experiment the GABAergic IPSCs and mIPSCs were abolished with gabazine (50 µM) and the glycinegic IPSCs and mIPSCs were abolished with strychnine (1 µM). α-Bungarotoxin (α-BgtTX, 100 nM) was used to block α7-subunit-containing nicotinic receptors and dihydro-β-erythrodine (DHβE) at a concentration of 3 µM was used to selectively block a4b2 nicotinic receptors (Alkondon and Albuquerque 1993). All drugs were purchased from Sigma Aldrich (St. Louis, MO).

Analysis of action potential–dependent IPSCs and TTX-insensitive mIPSCs were performed using MiniAnalysis (Synaptosoft, version 4.3.1) with a minimal acceptable amplitude of GABAergic or glycinegic IPSCs at 20 pA and that of the mIPSCs at 15 pA. Results are presented as means ± SE and statistically compared with paired and unpaired Student’s t-tests when appropriate. Significant difference was set at P < 0.05.

RESULTS

Application of neostigmine (10 µM), an acetylcholinesterase inhibitor, significantly increased the frequency of both GABAergic and glycinegic IPSCs in cardiac vagal neurons. The data from typical GABA and glycine experiments are shown in Fig. 1, A and B, respectively, while the summary data for the GABAergic and glycinegic experiments are illustrated in Fig. 1, C and D, respectively. Neostigmine (10 µM) significantly increased the GABAergic IPSC frequency in cardiac vagal neurons from 1.7 ± 0.1 to 3.5 ± 0.7 Hz (P < 0.05, n = 6) but did not significantly alter the amplitude of the GABAergic IPSCs or the holding current in cardiac vagal neurons. Neostigmine (10 µM) also significantly increased the glycinegic IPSC frequency in cardiac vagal neurons from 3.0 ± 0.6 to 4.9 ± 0.7 Hz (P < 0.05, n = 6) but did not significantly alter the amplitude of the glycinegic IPSCs or the holding current.

As shown from a representative experiment in Fig. 2A, exogenous application of nicotine (1.0 mM) increased both the frequency and amplitude of GABAergic IPSCs in premotor cardiac vagal neurons but did not change the holding current. The results from one experiment, as well as the summary data from 13 neurons, are shown in Fig. 2B, which also illustrates that nicotine (1.0 mM) evokes an increase in the frequency of GABAergic IPSCs from 4.1 ± 0.4 to 8.8 ± 0.6 Hz (P < 0.001), and the amplitude of GABAergic IPSCs increased from 43.6 ± 3.4 to 60.4 ± 5.4 pA (P < 0.001) in premotor cardiac vagal neurons.

To test whether the nicotine-evoked increase in GABAergic frequency and amplitude was dependent on α7-subunit–containing nicotinic receptors, the neurons were exposed to a second application of nicotine (1.0 mM) in the presence of the...
FIG. 1. In a typical experiment (A), application of neostigmine (10 μM), an acetylcholinesterase inhibitor, significantly increased the frequency of GABAergic inhibitory postsynaptic currents (IPSCs) in cardiac vagal neurons. Neostigmine (10 μM) also significantly increased the frequency of glycinergic IPSCs in a typical experiment (B). The time courses for these experiments are shown in C and D, respectively, as well as the summary data. Neostigmine (10 μM) significantly increased the GABAergic IPSC frequency in cardiac vagal neurons from 1.7 ± 0.1 to 3.5 ± 0.7 Hz (P < 0.05, n = 6) and increased the glycinergic IPSC frequency in cardiac vagal neurons from 3.0 ± 0.6 to 4.9 ± 0.7 Hz (P < 0.05, n = 6). Neostigmine had no significant effect on either GABAergic or glycinergic IPSC amplitudes (29 ± 2 vs. 37 ± 5 pA, P > 0.05; 98 ± 14 versus 99 ± 14 pA, P > 0.05, respectively) or holding currents (−82 ± 27 versus −99 ± 34 pA, P > 0.05; −166 ± 37 vs. −212 ± 41 pA, P > 0.05, respectively).
selective \( \alpha_7 \) nicotinic receptor antagonist \( \alpha\)-BgTx. The second application of nicotine in the presence of \( \alpha\)-BgTx was indistinguishable from the first application (Fig. 2C). These experiments also demonstrate that repetitive nicotine-evoked responses (with 20 min between nicotine application) could be obtained with brief (10–20 s) focal application of nicotine (1.0 mM). To ensure the lack of effect with \( \alpha\)-BgTX was not dependent on the sequence of application, \( \alpha\)-BgTX was applied during the first application of nicotine in some experiments. The nicotine-evoked responses in the presence of \( \alpha\)-BgTX were not different from control responses in the absence of \( \alpha\)-BgTX (\( P > 0.05 \)).

Nicotine also increased both the frequency and amplitude of glycinergic IPSCs. As shown from a representative experiment in Fig. 3A, nicotine (1.0 mM) increased both the frequency and amplitude of glycinergic IPSCs in premotor cardiac vagal neurons. The time course from one experiment is shown in Fig. 3A, and the results from 16 neurons are shown in Fig. 3B. Nicotine increased the glycinergic IPSC frequency from \( 3.3 \pm 0.5 \) to \( 7.3 \pm 1.0 \) Hz (\( P < 0.001 \)), and the amplitude was increased from \( 49.7 \pm 3.3 \) to \( 65.9 \pm 3.7 \) pA (\( P < 0.001 \)) in premotor cardiac vagal neurons. Similar to the lack of effect with GABAergic IPSCs, the selective \( \alpha_7 \) nicotinic receptor antagonist \( \alpha\)-BgTX had no effect of the nicotine-evoked increase in glycinergic frequency or amplitude (Fig. 3C).

In contrast the nicotine-evoked facilitation of both GABAergic and glycinergic IPSCs was abolished by DH\( \beta \)E at a concentration of 3 \( \mu \)M. As shown in a representative experiment in Fig. 4A, nicotine (0.1 mM) evoked an increase in both the frequency and amplitude of the GABAergic IPSCs, and these responses were abolished by 3 \( \mu \)M DH\( \beta \)E. The summary data from seven neurons is shown in Fig. 4B.

The nicotine-evoked facilitation in glycinergic IPSCs was also sensitive to DH\( \beta \)E. As shown in a representative example in Fig. 5A, nicotine (0.1 mM) evoked an increase in both frequency and amplitude of glycinergic IPSCs, and this nicotine-evoked facilitation was prevented by 3 \( \mu \)M DH\( \beta \)E. The results from nine neurons are summarized in Fig. 5B.

To test whether the site of action of nicotine could be on the GABAergic and glycinergic presynaptic terminals, TTX was applied to block action potential–dependent events, and TTX-insensitive GABAergic and glycinergic mIPSCs were examined. Nicotine (0.1 mM) evoked an increase in GABA mini-frequency, but did not alter GABA mini-amplitude, as shown in Fig. 6A. These experiments also demonstrate that repetitive nicotine-evoked responses (with 20 min between nicotine application) could be obtained with brief (10–20 s) focal application of nicotine (1.0 mM). To ensure the lack of effect with \( \alpha\)-BgTX was not dependent on the sequence of application, \( \alpha\)-BgTX was applied during the first application of nicotine in some experiments. The nicotine-evoked responses in the presence of \( \alpha\)-BgTX were not different from control responses in the absence of \( \alpha\)-BgTX (\( P > 0.05 \)).

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in a typical example in Fig. 6, A and B. The nicotine-evoked increase in GABA mini-frequency was abolished by 3 μM DHβE, as shown in a representative example (Fig. 6B) and in the average results from seven neurons (Fig. 6C). Similar to the GABAergic mIPSCs, in the presence of TTX nicotine (0.1 mM) evoked an increase in the frequency, but not amplitude of glycinergic mIPSCs, as shown in Fig. 7, A and B. DHβE prevented the nicotine-evoked increase in glycinergic mini-frequency (Fig. 7, B and C).

DISCUSSION

There are four major results from this study. Premotor cardiac vagal neurons receive glycinergic input. Application of neostigmine (10 μM), an acetylcholinesterase inhibitor, significantly increased the frequency of both GABAergic and glycinergic IPSCs to cardiac vagal neurons, indicating endogenous cholinergic activity facilitates GABAergic and glycinergic neurotransmission to cardiac vagal neurons in the nucleus ambiguus. Nicotine increases the frequency and amplitude of both glycinergic and GABAergic IPSCs, which were not diminished by the α7-subunit nicotinic blocker α-BgTX, but were abolished by the nicotinic antagonist DHβE at a concentration (3 μM) specific for α4β2 nicotinic receptors (Alkondon and Albuquerque 1993). The fourth major result from this study is that, in the presence of TTX, nicotine increased the frequency, but not the amplitude of both glycinergic and GABAergic mIPSCs, and this nicotine-mediated facilitation was also abolished by DHβE (3 μM).

The observation that premotor cardiac vagal neurons receive glycinergic input is supported by both anatomical and in vivo microinjection studies. Microinjection of glycine into the nucleus ambiguus has been shown to elicit tachycardia in spinal rats presumably by disinhibition:inhibition of these cardioinhibitory neurons (Chitravanshi et al. 1991). In addition premotor cardiac vagal neurons have been found to be densely innervated by fibers immunoreactive for glycine (Batten 1995).

There have been only two previous reports on the effect of nicotine on glycinergic synaptic transmission. In the rat, sym-
pathetic preganglionic neurons nicotinic antagonists inhibit glycinergic IPSCs, suggesting there may be an endogenous activation of nicotinic receptors that facilitates glycinergic neurotransmission to these sympathetic spinal cord neurons (Dun and Mo 1989). Also in the rat spinal cord nicotine has been shown to increase the frequency, but not amplitude of glycinergic mIPSCs, and the nicotine-mediated increase in glycinergic mini-frequency was not altered by α-BgTX, but was blocked by the nicotinic antagonist DHβE (Kiyosawa et al. 2001). The block of nicotinic responses by DHβE but not by α-BgTX is identical to the results in this study.

Nicotine has been shown in many other studies to enhance GABAergic neurotransmission, but one controversial issue is whether the nicotinic facilitation of GABAergic activity is

FIG. 4. DHβE (3 μM) abolished the nicotine-evoked facilitation of GABAergic neurotransmission as shown in a typical experiment (A) and the summary data from 7 experiments (B). In control experiments nicotine (0.1 mM) increased the frequency of GABAergic IPSCs from 4.1 ± 0.7 to 7.5 ± 0.8 Hz (P < 0.05) and increased the amplitude from 41.5 ± 4.1 to 53.1 ± 5.1 pA (P < 0.01). GABAergic IPSC responses to reapplication of nicotine in the presence of DHβE were abolished; both the changes in frequency (from 4.5 ± 0.6 to 5.0 ± 0.8 Hz) and amplitude (from 38.6 ± 3.5 to 43.8 ± 4.1 pA) were not significant (P > 0.05).

FIG. 5. DHβE (3 μM) inhibited the effects of nicotine on glycinergic IPSCs as shown in a typical experiment (A) and the summary data from 9 experiments (B). During control experiments nicotine (0.1 mM) increased the frequency of glycinergic IPSCs from 2.6 ± 0.5 to 5.4 ± 0.6 Hz (P < 0.001) and increased the amplitude from 50.9 ± 5.4 to 64.9 ± 5.3 pA (P < 0.05). The glycinergic IPSC responses to reapplication of nicotine were blocked in the presence of DHβE; frequency was not changed significantly (2.9 ± 0.6 to 3.2 ± 0.7 Hz) and amplitude was also not significantly altered (48.5 ± 4.5 to 46.9 ± 3.6 pA, P > 0.05).
“preterminal” and dependent on action potential generation in the GABAergic neuron or whether nicotine can act at the presynaptic terminal to enhance GABAergic neurotransmission independent of an action potential. In the ventral tegmental area the nicotinic facilitation of GABAergic neurotransmission is blocked by TTX, suggesting that nicotinic receptors are not sufficiently present on the presynaptic terminal to alter transmitter release, but presumably act by altering the action potential or other TTX-sensitive activity in the GABAergic neuron (Mansvelder et al. 2002). Similar results have been obtained in GABAergic neurotransmission to the rat interpeduncular nucleus, cerebellar Purkinje cells, dorsal motor nucleus, and CA1 neurons of the hippocampus (Alkondon et al. 1997; Bertolino et al. 1997; Lena et al. 1993; McMahon et al. 1994). However, in another study of the rat hippocampus, as well as in the mouse amygdala, the nicotinic enhancement of GABAergic neurotransmission occurred in the presence of TTX, suggesting that nicotine can act at the presynaptic terminal to facilitate GABA release (Barazangi and Role 2001; Fisher et al. 1998; Radcliffe et al. 1999). The results of this study demonstrate that nicotine can facilitate GABAergic and glycineergic neurotransmission in the presence of TTX, indicating that nicotine can enhance inhibitory neurotransmission to cardiac vagal neurons independent of action potential activity by activating nicotinic receptors present on the GABAergic and glycineergic presynaptic terminals.

The nicotine-evoked increase in both GABAergic and glycineergic IPSC amplitude could be due to changes in the activity of the preceding neurons, such as changes in the action potential waveform to alter neurotransmitter release, as well as augmentation of the postsynaptic inhibitory responses. Nicotine-mediated increases in GABAergic IPSC amplitude has also been observed in other work (Covernton and Lester 2002). However, since nicotine evoked an increase in GABAergic and glycineergic mini-frequency, but did not alter their amplitude, a more likely explanation for the increase in IPSC amplitude is that nicotine may be evoking summation of IPSCs by either recruitment of additional GABAergic and glycineergic neurons or summation of increased activity of previously active inhibitory neurons.

Interestingly, the nicotinic facilitation of both inhibitory GABAergic and glycineergic neurotransmission to cardiac vagal neurons is insensitive to α-BgTX and can be abolished by DHβE at a concentration (3 μM) specific for αβ2 nicotinic receptors (Alkondon and Albuquerque 1993), but the nicotinic facilitation of glutamatergic neurotransmission to cardiac vagal neurons is blocked by the α7-subunit nicotinic antagonist α-BgTX (Neff et al. 1998a). The heterogeneous profile of nicotinic enhancement of inhibitory neurotransmission by αβ2 nicotinic receptors and excitatory glutamatergic neurotransmission by α7-subunit–containing nicotinic receptors has also been observed in other neurons (Guo et al. 1998; Mansvelder et al. 2002).

The GABAergic results in this study is potentially complicated by the evidence that strychnine can inhibit α7 nicotinic receptors as well as glycine receptors (Matsubayashi et al.)
1998). This would cause an underestimate of the importance of α7 nicotinic receptors in the nicotinic facilitation of GABAergic inputs to cardiac vagal neurons. However the inhibition of α7 nicotinic receptors with 1 μM strychnine is only partial and is <10% (Matsubayashi et al. 1998). In other work from this laboratory (Neff et al. 1998a; Wang et al. 2001a) performed in the presence of 1 μM strychnine, activation of α7 nicotinic receptors facilitated glutamatergic neurotransmission to cardiac vagal neurons. It is therefore unlikely the complete absence of an effect with α-BgTX in this study is due to a prior antagonism with strychnine. Another potential complication of this study is that neostigmine can directly inhibit nicotinic receptors (Clarke et al. 1994; Nagata et al. 1997; Zheng et al. 1998). This would cause an underestimate of the endogenous nicotinic activation of inhibitory neurotransmission to cardiac vagal neurons. However the inhibition of α7 nicotinic receptors in facilitating both glycinergic and GABAergic neurotransmission to cardiac vagal neurons is likely small, since only neostigmine concentrations of 30 μM or larger significantly inhibited nicotinic receptors in rat CNS neurons, with an estimated IC₅₀ of 100 μM (Clarke et al. 1994).

In summary, endogenous cholinergic activity facilitates GABAergic and glycinergic neurotransmission to cardiac vagal neurons in the nucleus ambiguus. Activation of nicotinic receptors increases the frequency of both glycinergic and GABAergic miniature IPSCs as well as GABAergic and glycinergic miniature EPSCs to cardiac vagal neurons. These responses were not diminished by the α7-subunit nicotinic blocker α-BgTX, but were abolished by the nicotinic antagonist DHβE at a concentration specific for α4β2 nicotinic receptors. This nicotinic facilitation of inhibitory synaptic inputs to cardioinhibitory vagal neurons may be one mechanism for the increased heart rate prevalent in smokers. The nicotinic augmentation of inhibitory neurotransmission to cardiac vagal neurons may also be involved in the respiratory modulation of heart rate, since one cholinergic input to cardiac vagal neurons originates from neurons active in respiration (Irmaten et al. 2001).

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