Nicotine Enhances Presynaptic and Postsynaptic Glutamatergic Neurotransmission to Activate Cardiac Parasympathetic Neurons

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Abstract—Although peripheral cholinergic neurotransmission has long been known to play a pivotal role in the control of heart rate and blood pressure, recent evidence has suggested that central cholinergic mechanisms may be involved in the genesis of hypertension, anxiety, cardiorespiratory control, and, in particular, the respiratory modulation of heart rate. Yet, the sites, mechanisms, and receptor subtypes involved in the action of nicotine within the central nervous system are controversial. The present study demonstrates that nicotine has at least 3 sites of action to increase the activity of vagal cardiac neurons. Nicotine, but not muscarinic agonists, activates postsynaptic receptors and a depolarizing inward current in vagal cardiac neurons studied with the perforated patch-clamp technique in a visualized brain stem slice. In addition, nicotine acts at different presynaptic and postsynaptic sites to facilitate glutamatergic neurotransmission. Presynaptic nicotinic receptors increase the frequency of transmitter release and are sensitive to block by α-bungarotoxin. Nicotine also elicits a previously undescribed augmentation of postsynaptic non-NMDA currents. The presynaptic and postsynaptic receptors may prove to be future targets in the search for agonists to increase vagal cardiac activity and reduce the fatality associated with cardiac hyperexcitability and for antagonists to reduce cardiac vagal activity in pathological conditions associated with abnormally low heart rates and cardiac function such as sudden infant death syndrome. (Circ Res. 1998;83:1241-1247.)

Key Words: cardiac ■ parasympathetic ■ vagal ■ nicotine ■ brain stem ■ sudden infant death syndrome

It has long been known that nicotinic acetylcholine receptors are found throughout the peripheral nervous system and that these receptors play an important role in neurotransmission at the neuromuscular junction as well as in autonomic ganglia.1 Recent studies, however, have demonstrated that there are diverse types of nicotinic receptors within the central nervous system (CNS).2 Some of these nicotinic receptors, especially those that contain the α7 gene product, which are selectively blocked by α-bungarotoxin (α-Bgtx), are preferentially localized, and clustered, at presynaptic sites.3,5 These α-Bgtx–sensitive nicotinic receptors have a high permeability to calcium and have been shown to enhance synaptic transmission via presynaptic mechanisms.2,3,6–7

Nicotinic receptors within the CNS are thought to be responsible for many cardiorespiratory diseases, including sudden infant death syndrome (SIDS), which is the most common cause of deaths in infants between 1 month and 1 year of age.8 Although the origin of SIDS remains largely unknown, recent clinical studies suggest that maternal cigarette smoking is a major risk factor in SIDS9 and that an abnormality of cardiorespiratory control, particularly a centrally mediated slowing of the heart that precedes or accompanies apnea, is involved.10,11

Because the sites, mechanisms of action, and diverse receptor types of nicotine within the CNS are controversial and poorly understood, we examined in the present study the effects of nicotine on cardiac vagal neurons in the nucleus ambiguus. Heart rate in healthy individuals is determined primarily by the tonic and reflex control of these parasympathetic cardiac neurons that originate in the brain stem and directly project to the heart.1,12 We have found that there are different presynaptic and postsynaptic nicotinic receptors that have dramatic effects on glutamatergic neurotransmission and directly activate vagal cardioinhibitory neurons.

Materials and Methods

Animals were used in accordance with institutional and federal guidelines. In rats (6 to 12 days old), the heart was exposed in an initial surgery with a right thoracotomy, and rhodamine (XRITC, Molecular Probes) was injected into the pericardial sac and applied to the terminals of preganglionic parasympathetic cardiac neurons located mostly in the fat pads at the base of the heart. The animals were then allowed to recover for 3 to 7 days, after which the animals were anesthetized with methoxyflurane, killed by cervical dislocation, and the hindbrain was removed and placed for 1 minute in cold (0°C to 2°C) buffer composed of (in mmol/L) NaCl 140, KCl 5, CaCl2 2, glucose 5, HEPES 10 and continually gassed with 100% O2. The medulla was then cut in 250-μm-thick sections using a vi-
Slices were mounted in a perfusion chamber and submerged. The composition (in mmol/L) of the perfusate was NaCl 125, KCl 3, CaCl2 2, NaHCO3 26, dextrose 5, HEPES 5, constantly bubbled with 95% O2/5% CO2 and maintained at pH 7.4. Individual parasympathetic cardiac neurons were identified by the presence of the fluorescent tracer and were then imaged with differential interference contrast optics, infrared illumination, and infrared-sensitive video detection cameras to gain better spatial resolution and to visually guide and position the patch pipette onto the surface of the identified neuron. The pipette was advanced until a GΩ seal was obtained between the pipette tip and the cell membrane of the identified neuron. Access was obtained by allowing nystatin to form pores in the cell membrane. Picrotoxin (100 μmol/L), strychnine (1 μmol/L), prazosin (10 μmol/L), d-2-amino-5-phosphonovalerate (50 μmol/L), and tetrodotoxin (1 μmol/L) were added to the bath perfusate to prevent GABAergic, glycine, α1-adrenergic, and glutamatergic NMDA postsynaptic currents and to prevent activation of polysynaptic pathways, respectively. Patch pipettes were filled with a solution consisting of 130 mmol/L potassium gluconate, 10 mmol/L HEPES, 10 mmol/L EGTA, 1 mmol/L CaCl2, 1 mmol/L MgCl2, and 258 U/mL nystatin and had resistances of 2.5 to 3.5 MΩ. Pipette resistance and capacitance were compensated (>90%) before gaining intracellular access. Perforated patch access was monitored, and experiments were performed only after (10 to 20 minutes) a steady-state access resistance was obtained. Membrane resistances were calculated using voltage steps from −80 to −90 mV. To examine postsynaptic responses evoked by the spontaneous (not action potential evoked) release of transmitter, miniature synaptic events (minis) were recorded in the presence of tetrodotoxin, which blocks voltage-gated Na+ channels and action potential generation. Minis are thought to be the postsynaptic responses evoked by the spontaneous release of transmitter from a single presynaptic vesicle. Mini activity was recorded at a holding potential of −80 mV. Analysis of minis was conducted using Axograph (Axon Instruments) software, which automatically detects spontaneous minis, with a detection threshold of 4 SD from baseline noise. The detection algorithm uses a sliding template, as follows: \( f(t) = (1 - \exp(-u\ \text{rise}) \times \exp(-t/\text{rise}) \times \exp(-t/\text{decay}), \) where \( t = \text{time}, \text{rise} = \text{activation time constant}, \) and \( \text{decay} = \text{decay time constant}. \) Statistical tests were performed using paired and unpaired \( t \) tests as appropriate. Data are presented as mean±SEM.

### Results

Acetylcholine (Ach, 1 μmol/L) was first applied to vagal cardiac neurons to test whether these neurons possess postsynaptic cholinergic receptors. Application of Ach evoked an inward current and a decrease in membrane resistance. To determine the postsynaptic cholinergic receptors that were activated, bethanechol (0.1 to 1.0 mmol/L), a muscarinic agonist, and nicotine (1 to 2 mmol/L) were applied. Bethanechol had no effect, and there was no evidence of a muscarinic M-type current in these neurons (Figure 1). In contrast, nicotine evoked a postsynaptic response indistinguishable from Ach, eliciting an inward current and a decrease in membrane resistance (Figure 1). The nicotinic inward current had a reversal potential that was extrapolated to 22.6±7.4 mV. Both Ach and nicotine could evoke repeated responses if the applications were separated by at least 15 to 20 minutes. Surprisingly, in some of these vagal cardiac neurons, the membrane resistance was greater, and the baseline current was less negative, for a short period after nicotine application than it was during the control period (Figure 1). This suggested to us that nicotine may have initially enhanced, and then transiently inhibited, spontaneously active excitatory synaptic activity.

To test whether nicotine has other synaptic sites of action that could influence vagal cardiac neurons, spontaneous minis were also examined. (Traces including minis are shown in Figure 2D.) These experiments were also conducted in the presence of tetrodotoxin (1 μmol/L) to block action potential generation and eliminate polysynaptic pathways, as well as action potentials from the soma of presynaptic neurons. Minis are thought to be the postsynaptic responses evoked by the spontaneous (not action potential evoked) release of transmitter from a single presynaptic vesicle. Nicotine elicited repeatable increases in the frequency of minis (Figure 2B). These results are consistent with a presynaptic site of action and suggest that nicotine increases the excitability of presynaptic terminals and more vesicles are spontaneously released.

Surprisingly, the minis were also increased in amplitude, suggesting that a second mechanism exists that acts to augment the efficacy of synaptic transmission (Figure 2C and 2D). It is unlikely that the increase in mini amplitude is due to recruitment of a different population of vesicles, because...
the amplitude histogram of these events (Figure 2E) does not indicate that there is a polymodal distribution of mini amplitudes. The increase in mini amplitude could be caused by at least 2 additional mechanisms. One possibility is that the increase in mini amplitude is simply due to summation of nearly simultaneous minis. This would be a likely mechanism if the increase in mini amplitude is always accompanied by an increase in mini frequency. Another possibility is that a second mechanism exists to increase mini amplitude, and this mechanism is independent of changes in mini frequency.

To distinguish between these possibilities, α-Bgtx (0.1 μmol/L), which blocks the α7 gene product of the Ach nicotinic receptor, was applied. α-Bgtx selectively inhibited the presynaptic increase in transmitter release probability (Figure 3B), without altering the increase in mini amplitude (Figure 3C through 3E) or the direct postsynaptic responses (Figure 3A). The nicotinic receptors that, when activated, increase the frequency of synaptic release, and can be blocked by α-Bgtx, are likely located presynaptically. In contrast, the nicotinic receptors responsible for the increasing mini amplitude, as well as the long-lasting ligand-gated response, were not blocked by α-Bgtx. Given that the increase in mini amplitude persisted even when the increase in mini frequency was blocked, summation is very unlikely to be responsible for the increase in mini amplitude. Because the content of transmitter in single vesicles is not thought to be easily modulated, an increase in mini amplitude (independent of changes in mini frequency) is likely due to postsynaptic facilitation of receptor activation.

To test whether these mini synaptic events were due to glutamatergic synaptic activity, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 150 μmol/L), a non-NMDA channel blocker, was applied. CNQX completely inhibited the minis (Figure 4B through 4D) and also inhibited, but did not block, the long-lasting inward current (Figure 4A). This experiment not only confirms that the minis are due to glutamatergic synaptic activity but also that a third, and long-lasting, effect of nicotine is the activation of a ligand-gated receptor and an inward current that is, at least partly, independent of postsynaptic glutamatergic receptors.

To identify the postsynaptic receptors responsible for the increase in mini amplitude, and the direct postsynaptic response, curare (10 μmol/L) was applied (Figure 5). Curare is a less specific nicotinic antagonist that, in addition to blocking the α7 product, also blocks nicotinic receptors that are likely to be composed of the other gene products. As expected, curare blocked all of the nicotine responses, including the increase in mini frequency and amplitude (Figure 5B through 5E) and the postsynaptic inward current (Figure 5A).

**Discussion**

The neural control of the cardiovascular and respiratory systems are highly interrelated. These cardiorespiratory interactions are mediated by synaptic pathways within the CNS and are mediated largely, if not entirely, via the vagal innervation of the heart. In each respiratory cycle, the heart beats more rapidly during inspiration and slows during postinspiration and expiration (often referred to as respiratory sinus arrhythmia). The respiratory system also influences...
heart rate by modulating the baroreceptor and chemoreceptor input to cardiac vagal neurons. In animals and humans, the baroreceptor and chemoreceptor reflexes are inhibited during inspiration and are facilitated during postinspiration and expiration or during a maintained phase of postinspiration and apnea. As another example, stimulation of sensory laryngeal receptors evokes a prolonged apnea and a maintained and dramatic decrease in heart rate. This laryngeal reflex can be so exaggerated in newborns that it can lead to death in neonatal animals and has been suggested as a possible cause of SIDS. It is also worth noting that one of the largest risk factors associated with SIDS is maternal cigarette smoking, as well as postnatal exposure to nicotine.

Ach receptors within the CNS are likely to be involved in respiratory modulation of heart rate, because centrally acting, but not peripherally acting, cholinergic antagonists reduce respiratory sinus arrhythmia in humans. However, the respiratory phase in which Ach is presumably involved and the neurons responsible for this modulation are unknown. Cardiac vagal neurons recorded in vivo receive inhibitory synaptic input during inspiration, which is then followed by a rapid depolarization caused by excitatory synaptic input during postinspiration. Ach microinjected into the nucleus ambiguus in vivo has been shown to inhibit cardiac vagal activity in one study but excite cardiac vagal neurons in another study. Consistent with the excitatory action of Ach, cholinesterase inhibitors administered centrally decrease heart rate and increase the baroreflex control of heart rate. This augmentation is prevented by nicotinic antagonists.

These conflicting studies are difficult to interpret, because microinjections can alter the presynaptic and postsynaptic activity of many heterogeneous neurons, given that the nucleus ambiguus is composed of not only cardiac vagal but also gastrointestinal and ventral respiratory group neurons. The present study conclusively demonstrates that nicotine has at least 3 direct sites of action that act to increase the activity of vagal cardiac neurons and thereby decrease heart rate and depress cardiac function.

The presence of a direct ligand-gated excitatory postsynaptic current activated by nicotine in cardiac vagal neurons is somewhat surprising, given the greater density of muscarinic, rather than nicotinic, postsynaptic receptors in the CNS. However, there are some other notable exceptions, including nicotinic activation of a nonspecific cation conductance in Renshaw cells, as well as neurons in the nucleus tractus solitarius, medial habenula, and dorsal motor nucleus of the vagus. The nicotine-activated currents observed in vagal cardiac neurons, as seen in the present study, are nearly identical to the currents observed in other neurons. It is interesting to note that the neurons possessing postsynaptic nicotinic receptors are either known to be, or could be potentially, cholinergic neurons. This raises the possibility that these receptors are involved in some form of autostimulation.

In addition to the direct postsynaptic response, nicotine activates heterogeneous nicotinic receptors at both presynaptic and postsynaptic sites to facilitate glutamatergic neurotransmission in terminals surrounding vagal cardiac neurons.
The presynaptic nicotinic receptors are sensitive to block by α-Bgtx and therefore are likely to contain the α7 gene product of the nicotinic receptor. This gene product confers a large permeability to calcium, significantly larger than even NMDA receptors, suggesting that these receptors would likely play a significant role in the frequency of transmitter release, as shown by the increase in mini frequency demonstrated in the present study. In addition, nicotine has been shown to increase the frequency of transmitter release from glutamatergic synapses surrounding interpeduncular and sympathetic ganglia.

In cardiac vagal neurons, nicotine elicits a previously undescribed enhancement of postsynaptic non-NMDA glutamatergic receptors. Other experiments that have attempted to identify the nicotinic subtypes and mechanisms responsible for this facilitation have not yet been successful. Using the perforated patch configuration, which preserves intracellular second messenger responses, is apparently not required, because whole cell recordings did not preferentially inhibit the increase in mini amplitude evoked by nicotine. Other nicotinic antagonists (eg, dihydro-β-erythroidine and mecamylamine) did not preferentially inhibit the postsynaptic increase in mini amplitude. Additional work using specific antagonists for other nicotinic subtypes will be necessary to isolate the mechanisms responsible for postsynaptic facilitation of non-NMDA receptors.

As discussed previously, a likely neurotransmitter involved in the respiratory-modulated rhythm of heart rate is Ach. Some postinspiratory neurons, such as the superior laryngeal motor neurons, synthesize and release Ach at their peripheral

Figure 4. CNQX (50 μmol/L), a non-NMDA channel antagonist, blocked the minis. CNQX inhibited, but did not block completely, the inward current evoked by nicotine (A). However, CNQX was completely effective in blocking minis before and during nicotine application (B through D). Mean data are from 6 cardiac vagal neurons.
and axon collateral synapses. These neurons are also colocalized with cardiac vagal neurons in the nucleus ambiguus and have many axon collaterals within the nucleus ambiguous. One possibility is that postinspiratory cholinergic neurons influence cardiac vagal neurons via 3 independent mechanisms. One site of action may be via direct activation of postsynaptic ligand-gated nicotinic channels in cardiac vagal neurons, which can act to depolarize and excite cardiac vagal neurons during postinspiration. An additional site of action could be presynaptic and involve the observed nicotinic facilitation of presynaptic glutamatergic synaptic terminals demonstrated in the present study. A third action of Ach could be to facilitate the responses in postsynaptic non-NMDA receptors on the release of glutamate from other neurons. These latter 2 effects may constitute mechanisms by which respiratory inputs gate, or facilitate, the baroreflex during postinspiration.

In conclusion, the present study demonstrates that nicotine has at least 3 sites of action to increase the activity of vagal cardiac neurons. Nicotine, but not muscarinic agonists, activates postsynaptic receptors and a depolarizing inward current in vagal cardiac neurons studied with the perforated patch-clamp technique in a visualized brain stem slice. In addition, nicotine acts at different presynaptic and postsynaptic sites to facilitate glutamatergic neurotransmission. Presynaptic nicotinic receptors increase the frequency of transmitter release and are sensitive to block by α-Bgtx. Nicotine also elicits a previously undescribed augmentation of postsynaptic non-NMDA currents. Presynaptic and postsynaptic nicotinic antagonists may prove to be future targets to reduce cardiac vagal activity in pathological conditions associated with abnormally low heart rates and cardiac function, such as SIDS, and agonists may be beneficial to increase vagal cardiac activity and reduce the fatality associated with cardiac hyperexcitability.

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References


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