

# Agatoxin-IVA-Sensitive Calcium Channels Mediate the Presynaptic and Postsynaptic Nicotinic Activation of Cardiac Vagal Neurons

JIJANG WANG, MUSTAPHA IRNATEN, AND DAVID MENDELOWITZ

Department of Pharmacology, George Washington University, Washington, DC 20037

Received 7 July 2000; accepted in final form 2 October 2000

**Wang, Jijiang, Mustapha Irnaten, and David Mendelowitz.** Agatoxin-IVA-sensitive calcium channels mediate the presynaptic and postsynaptic nicotinic activation of cardiac vagal neurons. *J Neurophysiol* 85: 164–168, 2001. Whole cell currents and miniature glutamatergic synaptic events (minis) were recorded in vitro from cardiac vagal neurons in the nucleus ambiguus using the patch-clamp technique. We examined whether voltage-dependent calcium channels were involved in the nicotinic excitation of cardiac vagal neurons. Nicotine evoked an inward current, increase in mini amplitude, and increase in mini frequency in cardiac vagal neurons. These responses were inhibited by the nonselective voltage-dependent calcium channel blocker Cd (100  $\mu$ M). The P-type voltage-dependent calcium channel blocker agatoxin IVA (100 nM) abolished the nicotine-evoked responses. Nimodipine (2  $\mu$ M), an antagonist of L-type calcium channels, inhibited the increase in mini amplitude and frequency but did not block the ligand gated inward current. The N- and Q-type voltage-dependent calcium channel antagonists conotoxin GVIA (1  $\mu$ M) and conotoxin MVIIC (5  $\mu$ M) had no effect. We conclude that the presynaptic and postsynaptic facilitation of glutamatergic neurotransmission to cardiac vagal neurons by nicotine involves activation of agatoxin-IVA-sensitive and possibly L-type voltage-dependent calcium channels. The postsynaptic inward current elicited by nicotine is dependent on activation of agatoxin-IVA-sensitive voltage-dependent calcium channels.

## INTRODUCTION

The control of heart rate and cardiac function is determined primarily by cardiac vagal neurons that are located in the nucleus ambiguus (NA) and the dorsal motor nucleus (DMNX) of the vagus (Izzo et al. 1993; Standish et al. 1994, 1995; Taylor et al. 1999). These neurons dominate the neural control of heart rate under normal conditions and also determine the prognosis of many pathological cardiac challenges (Vanili and Schwartz 1990). In each respiratory cycle, the heart beats more rapidly in inspiration and slows during postinspiration and expiration (referred to as respiratory sinus arrhythmia). Cardiorespiratory interactions do not seem to occur between sensory neurons at their first central synapses in the nucleus tractus solitarius (NTS), suggesting cardiorespiratory interactions occur later in the reflex pathways perhaps within the nucleus ambiguus (Mifflin et al. 1988). With the discovery of diverse types of nicotinic acetylcholine receptors (nAChRs) in the CNS, particularly the evidence that nAChRs exist in synaptic terminals in NA and DMNX (Winzer-Serhan and Leslie 1997),

acetylcholine as a neurotransmitter is now suggested to be responsible for the respiratory modulation of heart rate and may be involved in many cardiorespiratory diseases, including sudden infant death (Feldman and Buccafusco 1993; Florez et al. 1990; Loewy and Spyer 1990; Mallard et al. 1999; Panigrahy et al. 1997; Slotkin 1998).

Previous studies from this laboratory have described the activation of cardiac preganglionic vagal neurons by nicotine and acetylcholine (Mendelowitz 1998; Neff et al. 1995, 1998a). This activation involves presynaptic nAChRs that increase the frequency of spontaneous glutamatergic miniature synaptic events (minis) and postsynaptic nAChRs that enhance non-*N*-methyl-D-aspartate (NMDA) currents and evoke a direct ligand gated inward current. The increase of mini frequency was blocked by  $\alpha$ -bungarotoxin ( $\alpha$ -Bgtx), a selective antagonist of nAChRs that contain the  $\alpha$ -7 gene product (Clarke 1992; Couturier et al. 1990), suggesting the existence of these types of nAChRs in the presynaptic terminals of neurons that project to cardiac vagal neurons.

The mechanisms by which  $\alpha$ -Bgtx sensitive nAChRs increase the frequency of spontaneous release of transmitter are unclear.  $\alpha$ -Bgtx-sensitive nAChRs have a high permeability to calcium, are preferentially localized, and are clustered at presynaptic sites (Amador and Dani 1995; Castro and Albuquerque 1995; Clarke 1992; Couturier et al. 1990; Zhang et al. 1996). One possibility is that the calcium influx elicited from activation of the nAChR channel is sufficient to cause spontaneous transmitter release. Another possibility is that the spontaneous transmitter release depends on nAChR-evoked presynaptic depolarization and subsequent activation of presynaptic voltage-dependent calcium channels (VDCCs). This second mechanism has been shown to be involved in the enhancement of spontaneous GABA release from neuron terminals within chick lateral spiriform and ventral lateral geniculate (Tredway et al. 1999). It is also not known if VDCC are involved in the postsynaptic enhancement of non-NMDA currents or the direct ligand-gated inward current elicited by nicotine in cardiac vagal neurons.

The present study investigates whether VDCCs play a role in the presynaptic and postsynaptic nicotinic activation of cardiac preganglionic vagal neurons and the identity of the specific VDCC subtype(s) involved. This study indicates that nicotine-evoked increase in presynaptic transmitter release does involve

Address for reprint requests: D. Mendelowitz, Dept. of Pharmacology, George Washington University, 2300 Eye St. N.W., Washington, DC 20037 (E-mail: dmendel@gwu.edu).

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presynaptic VDCCs, and among the subtypes, agatoxin-IVA-sensitive channels predominate with L type channels playing a minor role. Unexpectedly, the same VDCC subtypes are responsible for the postsynaptic enhancement of non-NMDA currents on activation of nAChRs.

## METHODS

Terminals of preganglionic vagal cardiac neurons are located mostly in the fat pads at the base of the heart in rats (Standish et al. 1994). In an initial surgery, rats of 6- to 12-days old were anesthetized with methoxyflurane and hypothermia and received a right thoracotomy. The heart was exposed, and rhodamine (XRITC, Molecular Probes) was injected into the pericardial sac. On the day of experiment (3–7 days later), the animals were anesthetized with methoxyflurane and killed by rapid cervical dislocation. All animal procedures were performed in compliance with the institutional guidelines at George Washington University and are 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 hindbrain was removed and placed for 1 min in cold (0–2°C) buffer of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 5 glucose, and 10 HEPES and was continually gassed with 100% O<sub>2</sub>. The medulla was then cut in sections of 250- $\mu$ m thickness using a vibratome. Slices were mounted in a perfusion chamber and submerged in the perfusate of following composition (in mM): 125 NaCl, 3 KCl, 2 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 5 dextrose, and 5 HEPES, constantly bubbled with gas (95% O<sub>2</sub>-5% CO<sub>2</sub>), and maintained at pH 7.4. Calcium channel antagonists were directly perfused into the chamber at the following concentrations. CdCl<sub>2</sub> (100  $\mu$ M) was dissolved directly into the perfusate; nimodipine was dissolved in ethanol for a stock solution of 2 mM, and diluted to a final concentration of 2  $\mu$ M. Conotoxin GVIA, agatoxin IVA, and conotoxin MVIIC were prepared immediately before use and their concentrations were 1  $\mu$ M, 100 nM, and 5  $\mu$ M, respectively.

Individual vagal cardiac neurons were identified by the presence of the fluorescent tracer. These identified vagal cardiac neurons were then imaged with differential interference contrast (DIC) 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 obtaining a seal over 1 G $\Omega$  between the pipette tip and the cell membrane of the identified neuron. The membrane under the pipette tip was then ruptured with a brief suction to obtain whole cell patch-clamp configuration, and the cell was voltage-clamped at a holding potential of –80 mV. Picrotoxin (100  $\mu$ M), strychnine (1  $\mu$ M), prazosine (10  $\mu$ M), D-2-amino-5-phosphonovalerate (50  $\mu$ M), and tetrodotoxin (TTX, 1  $\mu$ M) were infused to prevent GABAergic, glycinergic,  $\alpha_1$ -adrenergic, glutaminergic NMDA postsynaptic currents and to prevent polysynaptic pathways, respectively. The pipettes were filled with a solution consisting of (in mM) 130 K<sup>+</sup> gluconate, 10 HEPES, 10 EGTA, 1 CaCl<sub>2</sub>, and 1 MgCl<sub>2</sub>.

Nicotine (1  $\mu$ M) was pressure injected directly onto the neuron by a micropipette positioned directly above the neuron for 20–40 s. After application, a brief (approximately 10 s) negative pressure pulse was applied to limit any diffusion of nicotine out of the micropipette. The slice was then perfused with a calcium channel antagonist for 20 min. At the end of this 20-min period, nicotine was reapplied in the continued presence of the antagonist. A 20-min delay was used to minimize any desensitization of the cell by nicotine. Analysis of spontaneous postsynaptic events was performed using MiniAnalysis (Synaptosoft, version 4.3.1) with an amplitude threshold of 6 pA. Different groups of neurons were examined for each toxin. Responses to nicotine during simultaneous application of the toxin were statistically compared with the responses from control nicotine applications

using student's paired *t*-test. The data are presented as means  $\pm$  SE, with one asterisk signifying differences of  $P \leq 0.05$ , and two asterisks for  $P \leq 0.01$ .

## RESULTS

Nicotine evoked an inward current in cardiac vagal neurons as well as dramatic increases in both the frequency and the amplitude of the minis (Fig. 1, *left*). CdCl<sub>2</sub> inhibited, but did not completely abolish, this nicotinic activation of cardiac vagal neurons (Fig. 1, *right*). In the presence of CdCl<sub>2</sub>, nicotine did not induce any significant change in baseline current. Nicotine significantly increased mini frequency and amplitude when accompanied with CdCl<sub>2</sub>, but these responses were significantly reduced by 75.5 and 83.9%, respectively, compared with control responses.

Since the nonspecific VDCC blocker CdCl<sub>2</sub> inhibited the pre- and postsynaptic nicotine-evoked responses, we next examined which VDCCs were responsible for these nicotine-mediated changes. The specific P-type VDCC blocker  $\omega$ -agatoxin IVA (100 nM) completely abolished all presynaptic and postsynaptic nicotinic activation of cardiac vagal neurons.  $\omega$ -Agatoxin IVA (Fig. 2, *right*) blocked the nicotine-induced inward current, increases in mini frequency and mini amplitude by 98.2, 87.0, and 99.6%, respectively, compared with control responses (Fig. 2, *left*).

In contrast the specific L-type VDCC blocker nimodipine inhibited the nicotine-evoked increases in mini amplitude and

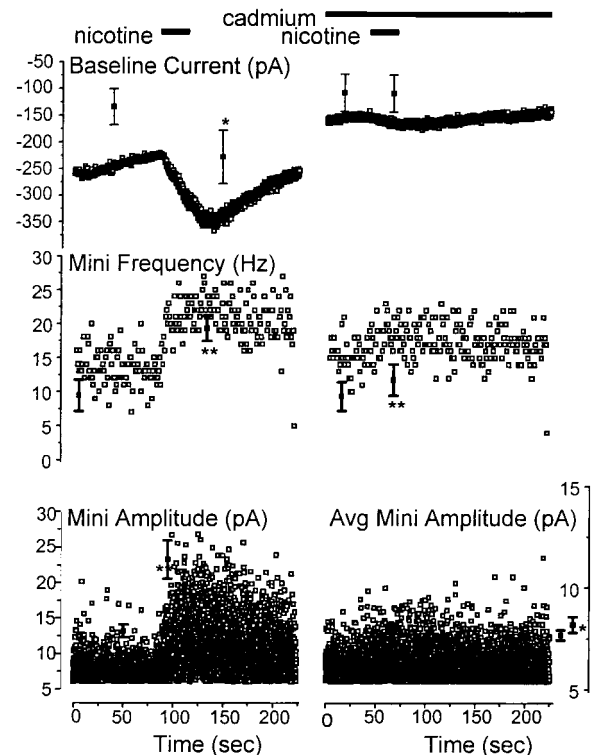


FIG. 1. *Left*: nicotine evokes an inward current, increase in mini frequency, and increase in mini amplitude in cardiac vagal neurons. *Right*: when Cd (100  $\mu$ M) is included in the perfusate, the nicotine-evoked inward current is blocked and the increase in mini frequency and amplitude are significantly inhibited ( $n = 8$ ). In this and all subsequent figures, the vertical axes for the typical experiments shown are shown on the *left*, and the vertical axes for the averaged data illustrated as solid squares  $\pm$  SE, when different, are shown on the *right*.

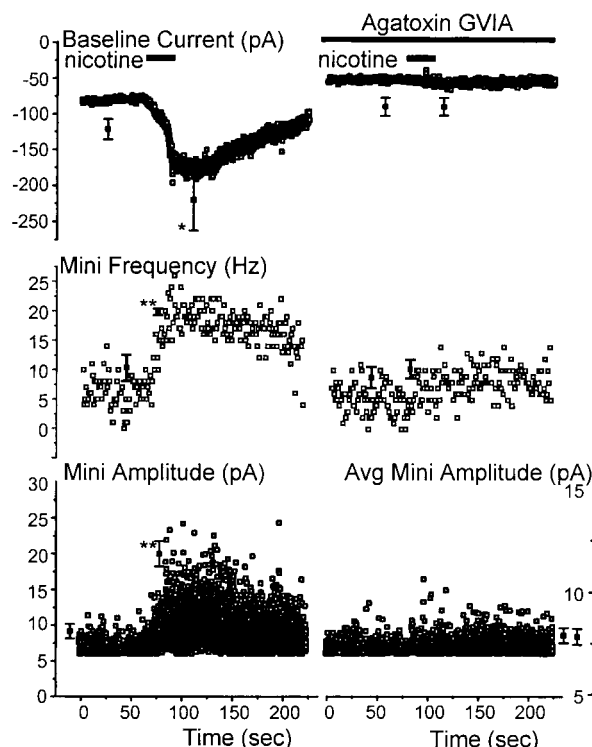


FIG. 2. The specific voltage-dependent calcium channel (VDCC) blocker  $\omega$ -Agatoxin IVA (100 nM) completely abolished all pre- and postsynaptic nicotinic activation of cardiac vagal neurons.  $\omega$ -Agatoxin IVA blocked the nicotine-induced inward current and increases in mini frequency and mini amplitude by 98.22, 86.99, and 99.64%, respectively (right), compared with control responses (left,  $n = 5$ ).

frequency but did not significantly inhibit the nicotine-elicited ligand-gated inward current (Fig. 3). The nicotine-evoked increases in mini frequency and amplitude were not statistically significant from baseline values when accompanied by nimodipine. However, the nicotine-evoked inward current during nimodipine remained statistically significant and was only attenuated by 46.6% from the control nicotine response.

$\omega$ -Conotoxin MVIIC and  $\omega$ -conotoxin GVIA did not alter the nicotinic activation of cardiac vagal neurons. In the presence of  $\omega$ -conotoxin MVIIC (5  $\mu$ M), the nicotine-induced increases of the frequency and the amplitude of the minis, as well as the inward current, were not significantly different from control responses. Similarly  $\omega$ -conotoxin GVIA (1  $\mu$ M) did not alter the pre- and postsynaptic nicotine-evoked responses. The effects of each calcium channel antagonist on the nicotinic activation of cardiac vagal neurons are summarized in Fig. 4.

## DISCUSSION

Recent studies of cardiac vagal neurons in the nucleus ambiguus (NA) have demonstrated that these neurons are intrinsically silent and do not exhibit spontaneous pacemaker like activity (Mendelowitz 1996). In agreement with these *in vitro* results, the relatively few *in vivo* studies that have successfully examined preganglionic cardiac neurons with extracellular electrodes have also found that most of these neurons (identified by antidromic stimulation) were silent (Giby et al. 1984). These results suggest that the tonic vagal activity that is normally present in unanesthetized animals depends on excitatory synaptic contacts to initiate and maintain vagal cardiac activity.

Two important transmitters that excite cardiac vagal neurons are glutamate and acetylcholine. One of the more functionally important pathways for blood pressure regulation is the glutamatergic pathway from the NTS (Neff et al. 1998b; Taylor et al. 1999; Willis et al. 1996). The NTS is the site of the first central synapse for visceral sensory neurons, including arterial baroreceptors, and the monosynaptic pathway from the NTS to cardiac vagal neurons most likely plays an essential role in cardiovascular reflex control. Acetylcholine is also an important transmitter innervating cardiac vagal neurons, and this input likely play an important role in cardiorespiratory interactions (Giby et al. 1984). Our previous work has shown that nicotine, but not muscarinic agonists, activates postsynaptic receptors and a depolarizing inward current in vagal cardiac neurons (Neff et al. 1998a). In addition, nicotine acts at different pre- and postsynaptic sites to facilitate glutamatergic neurotransmission (Mendelowitz 1998; Neff et al. 1998a). Presynaptic nicotinic receptors increase the frequency of transmitter release, and are sensitive to block by  $\alpha$ -bungarotoxin. Nicotine also elicits an augmentation of postsynaptic non-NMDA currents. These nicotinic responses may serve to directly depolarize cardiac neurons as well as augment the glutamatergic input to cardiac vagal neurons both pre- and postsynaptically during the postinspiratory phase of the respiratory cycle. These latter two effects may constitute mechanisms by which cholinergic respiratory neurons gate or facilitate the baroreflex during postinspiration.

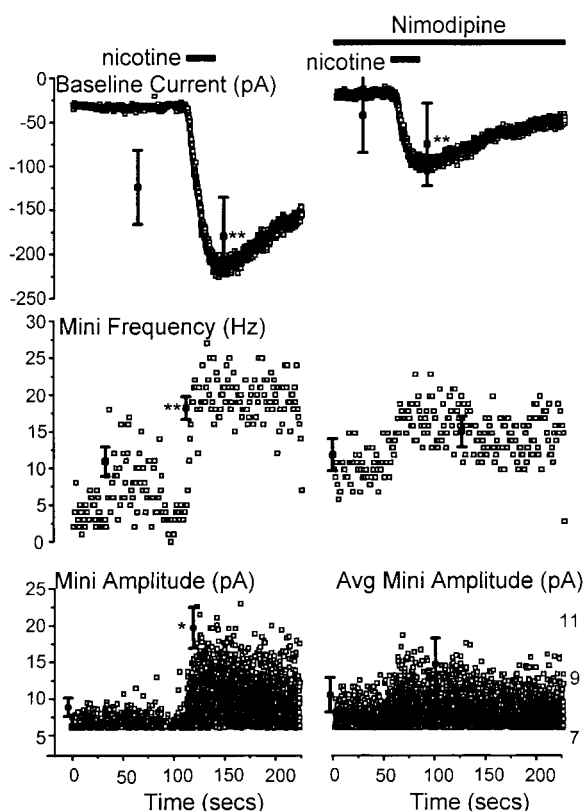


FIG. 3. Right: the L-type calcium channel blocker nimodipine (2  $\mu$ M) significantly attenuated the nicotine-evoked increases in mini frequency and amplitude but did not significantly alter the nicotine-elicited ligand-gated inward current. The nicotine-evoked increases in mini frequency and amplitude were blocked with nimodipine, but the nicotine-evoked inward current remained statistically significant and was only attenuated by 46.63% from the control response during nimodipine ( $n = 9$ ).



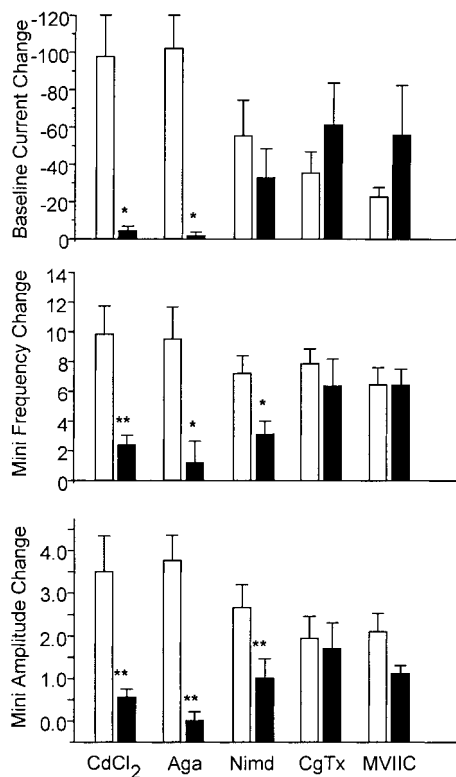


FIG. 4. Nicotine-evoked responses (□) were blocked by Cd (100  $\mu$ M,  $n = 8$ ; ■). The specific P-type VDCC blocker agatoxin IVA (100 nM,  $n = 5$ ) also blocked the inward current and increase in mini frequency and amplitude. The specific L-type VDCC blocker nimodipine (2  $\mu$ M,  $n = 9$ ) blocked the nicotine-evoked increase in mini frequency and amplitude but did not significantly alter the nicotine-evoked ligand-gated inward current.  $\omega$ -Conotoxin MVIIC (5  $\mu$ M,  $n = 8$ ) and  $\omega$ -conotoxin GVIA (1  $\mu$ M,  $n = 5$ ), specific blockers of the Q- and N-type VDCCs, respectively, did not alter the nicotinic activation of cardiac vagal neurons.

This work demonstrates that VDCCs play a critical role in mediating the nicotinic facilitation of glutamatergic responses both pre- and postsynaptically. The nonspecific VDCC blocker Cd and the specific VDCC blocker agatoxin IVA inhibited the nicotine-evoked increase in mini frequency. This suggests that although the presynaptic  $\alpha 7$  subunit containing nicotinic receptors is responsible for the increase in transmitter discharge and is highly permeable to calcium, the increase in mini frequency is dependent on concurrent activation of VDCCs, and in particular agatoxin-IVA-sensitive calcium channels. It therefore seems likely that activation of  $\alpha 7$  subunit containing presynaptic nicotinic receptors is sufficient to evoke depolarization at the presynaptic terminals to activate VDCCs but cannot independently increase presynaptic calcium to sufficient levels to evoke transmitter release.

The postsynaptic facilitation of non-NMDA currents and the direct ligand-gated inward current elicited by nicotine are also dependent on activation of VDCCs. The subtype of nicotinic receptors located postsynaptically in cardiac vagal neurons that are responsible for these postsynaptic responses are unknown. The postsynaptic nicotinic receptors in cardiac vagal neurons are insensitive to  $\alpha$ -bungarotoxin and therefore do not contain the  $\alpha 7$  subunit (Neff et al. 1998a). Surprisingly however, the postsynaptic nicotinic responses are also dependent on VDCCs and can be blocked by Cd as well as agatoxin IVA. This suggests that both the nicotinic facilitation of non-NMDA currents, as well as the direct postsynaptic inward currents,

require activation of VDCCs and in particular the agatoxin IVA-sensitive VDCC. It is possible that these nicotinic responses rely on a Ca-dependent or other second messenger that is only activated when postsynaptic VDCCs are at least partially open.

Nimodipine partially inhibited the nicotine-evoked increase in mini frequency and amplitude but did not significantly alter the direct inward current. One possibility is that L-type VDCCs are involved in enhancing glutamatergic transmission; but another possibility is that nimodipine directly inhibits these nicotinic receptors. Other studies have provided evidence that L-type VDCC antagonists may directly alter nicotinic receptors (Donnelly-Roberts et al. 1995). Since the subunit composition and the full pharmacological profile of the nicotinic receptors located postsynaptically in cardiac vagal neurons are unknown, we cannot rule out the possibility that nimodipine directly inhibits these nicotinic receptors. It is highly unlikely that the inhibition of the nicotinic responses with Cd or agatoxin are due to direct effects on the nicotinic receptors since the concentration of Cd and agatoxin used in this study (100  $\mu$ M and 100 nM, respectively) have been shown to have no effect on nicotinic receptors (Donnelly-Roberts et al. 1995).

It is somewhat surprising that the nicotine-evoked responses are blocked by agatoxin IVA but are unaltered by  $\omega$ -conotoxin MVIIC. In many neurons, studies have shown that agatoxin IVA and  $\omega$ -conotoxin MVIIC block the same P/Q type VDCCs (for review, see Meir et al. 1999). However, there are some neurons in which a population of VDCCs are blocked by agatoxin IVA but are insensitive to  $\omega$ -conotoxin MVIIC. For example, in crayfish nerve terminals (Wright et al. 1996), peptidergic neurons (Garcia-Colunga et al. 1999), and teleost retinal cone horizontal neurons (Pfeiffer-Linn and Lasater 1996), agatoxin IVA nearly completely blocked VDCCs while these VDCCs were not altered by  $\omega$ -conotoxin MVIIC. In humans, there may be developmental changes in the sensitivity of VDCCs to blockers since in a 10-mo-old child, but not in adults, hippocampal granule cells are agatoxin IVA sensitive but are insensitive to  $\omega$ -conotoxin MVIIC (Beck et al. 1997). It has recently been suggested that there is a much greater diversity of voltage-dependent calcium channel subtypes than previously recognized and can be distinguished with the L, N, P, Q, and R toxin-based classification (Burley and Dolphin 2000).

In summary, the nicotine-evoked inward current and both the pre- and postsynaptic enhancement of glutamatergic synapses in cardiac vagal neurons is dependent of activation of agatoxin-IVA-sensitive VDCCs. L-type, but not N- or  $\omega$ -conotoxin MVIIC-type, VDCCs may also be involved. It therefore seems likely that for nicotine to enhance glutamatergic pathways to cardiac vagal neurons, the presynaptic neurons need to depolarize sufficiently to activate VDCCs, and the VDCCs are also involved in the postsynaptic facilitation.

The technical assistance of K. Peterson, C. Evans, and A. Elibero is gratefully acknowledged.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-49965 and HL-59895.

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