Baro-Activated Neurons With Pulse-Modulated Activity in the Rat Caudal Ventrolateral Medulla Express GAD67 mRNA

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Schreihofer, Ann M. and Patrice G. Guyenet. Baro-activated neurons with pulse-modulated activity in the rat caudal ventrolateral medulla express GAD67 mRNA. J Neurophysiol 89: 1265–1277, 2003; 10.1152/jn.00737.2002. GABAergic neurons in the caudal ventrolateral medulla (CVLM) are believed to mediate the sympathetic baroreceptor reflex by inhibiting presympathetic neurons in the rostral ventrolateral medulla (RVLM). Accordingly, some CVLM neurons are activated by increased arterial pressure (AP; baro-activated), have activity strongly modulated by the AP pulse (pulse-modulated), and can be antidromically activated from the RVLM. This study examined whether baro-activated, pulse-modulated CVLM neurons are indeed GABAergic and examined their structures. We recorded extracellularly from 19 baro-activated, pulse-modulated CVLM neurons in chloralose-anesthetized rats. Most of these cells (13/19) were silenced by decreasing AP with nitroprusside, but some (6/19) remained active at low AP levels. They were also excited by phenyl biguanide (17/17) but inhibited by noxious tail pinch (8/11). Twelve baro-activated cells were filled with biotinamide and examined for expression of GAD67 mRNA. Because adjacent vagal motor neurons are also activated by increased AP, we examined choline acetyltransferase (ChAT) immunoreactivity. Most baro-activated cells (9/12) expressed high levels of GAD67 mRNA, the rest (3/12) displayed lower levels of GAD67 mRNA, but none showed ChAT immunoreactivity. In contrast, adjacent baro-inhibited CVLM cells had no GAD67 mRNA (n = 5) but were instead tyrosine hydroxylase immunoreactive (n = 7). Reconstruction of baro-activated CVLM neurons revealed axons that projected dorsomedially and rostrally with several axon collaterals. These data demonstrate the existence of GABAergic CVLM neurons with physiological characteristics expected of interneurons that mediate the sympathetic baroreceptor reflex. In addition, baro-activated GABAergic CVLM neurons appear to integrate several types of inputs and provide inhibition to multiple targets.

INTRODUCTION

The central pathway of the sympathetic baroreceptor reflex is believed to include GABAergic caudal ventrolateral medulla (CVLM) neurons that receive excitatory inputs from the nucleus tractus solitarius (NTS) and in turn project to presympathetic neurons in the rostral ventrolateral medulla (RVLM) (e.g., Blessing 1997; Chan and Sawchenko 1998; Sved and Gordon 1994). Axonal terminals from NTS neurons make synaptic contacts with the dendrites and somata of CVLM neurons that project toward the RVLM (Aicher et al. 1995). In addition, the CVLM contains GABAergic neurons that project to the RVLM, which express Fos following sustained increases in arterial pressure (AP) (Chan and Sawchenko 1998; Minson et al. 1997). Baroreflex-mediated changes in RVLM neuronal activity, sympathetic nerve activity (SNA), and AP are attenuated or prevented by inhibition of the CVLM, antagonism of glutamatergic receptors in the CVLM (Agarwal et al. 1990; Gordon 1987; Guyenet et al. 1987), or antagonism of GABAergic receptors in the RVLM (Sun and Guyenet 1985). In contrast, stimulation of the CVLM mimics the activation of baroreceptor afferents, by decreasing the activity of presympathetic RVLM neurons, SNA, and AP (Agarwal et al. 1989; Li et al. 1991; Masuda et al. 1991). Finally, the CVLM contains neurons with firing properties, suggesting they could be baroreceptor reflex interneurons. Namely, these neurons are activated by increased AP, display modulation of their activity in relation to the AP pulse, and project toward the RVLM (Agarwal and Calaresu 1991; Gieroba et al. 1992; Jeske et al. 1993; Terui et al. 1990). However, none of these studies have demonstrated that the baro-activated CVLM neurons are GABAergic, and the morphology of these neurons remains unknown. Therefore the present study sought to determine whether the CVLM neurons with the appropriate firing properties are indeed GABAergic and project to the RVLM. Individually recorded CVLM neurons were physiologically characterized and then filled with biotinamide for histological analysis. A preliminary version of this data was presented at the Experimental Biology meeting in 2001 (Schreihofer and Guyenet 2002).

METHODS

All experiments were performed on male Sprague–Dawley rats (250–350 g, Hilltop Laboratories, Scottsdale, PA) in accordance with National Institutes of Health and Institutional Animal Care and Use Guidelines. The University of Virginia Animal Research Committee approved all procedures and protocols.

Surgical preparation

Anesthesia was induced with 5% halothane. Surgical procedures were performed under halothane (1.7% in 100% O2) administered by a tracheal cannula. Catheters were placed in the right brachial artery and femoral vein to record AP and heart rate (HR) and to administer the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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drugs, respectively. An inflatable snare was wrapped around the subdia phragmatic aorta to produce rapid and reliable changes in upper body AP (Brown and Guyenet 1985). Splanchnic SNA was recorded as previously described (Schreihofer and Guyenet 2000; Schreihofer et al. 2000). The left splanchnic nerve was isolated via a retroperito neal approach, and the segment distal to the suprarenal ganglion was placed on two Teflon-coated silver wires that had been bared at the tip (250-μm bare diam., A-M Systems, www.a-msystems.com). The nerve and wires were embedded in a dental impression material (polyvinylsiloxane, Darby.Spencer.Mead Dental Supply, www.darbyspencermead.com), and the wound was closed around the exiting recording wires. For extracellular recordings of neurons in the CVLM, the rat was placed in a stereotaxic instrument with the incisor bar positioned 11 mm below the interaural line. The dorsal surface of the medulla was exposed via a limited craniotomy and the calamus scriptorius was visualized with the aid of a surgical microscope. On completion of surgical procedures, the halothane was replaced by α-chloralose (70 mg/kg iv of a 30 mg/ml solution in 3% sodium borate, with hourly supplements of one-third of the initial dose), and the rat was allowed to stabilize for 30 min. Rectal temperature (maintained at 37°C) and end-tidal CO₂ (maintained at 4.5–5.5%) were monitored throughout the experiment. Adequacy of anesthesia was periodically determined by lack of withdrawal response to firm toe pinch and absence of corneal reflex. Shortly before recording from CVLM units, the rats were paralyzed with pancuronium bromide (1 mg/kg iv, Elkins-Sinn, Cherry Hill, NJ).

Extracellular recording and juxtacellular labeling of neurons in the CVLM

In 25 chloralose-anesthetized, artificially ventilated rats the discharges of barosensitive neurons in the CVLM were recorded extracellularly as previously described (Schreihofer and Guyenet 1997; Schreihofer et al. 2000) using glass electrodes filled with 1.5 or 5% biotinamide (Molecular Probes, www.molecularprobes.com) in 0.5 M sodium acetate. Optimal electrode resistance for recording and labeling cells was 20–40 MΩ measured in vivo. Recordings were made with an intracellular amplifier in bridge mode (Axoclamp 2A, Axon Instruments, www.axon.com) to allow monitoring of action potentials during injection of current through the electrode. The CVLM was located using stereotaxic coordinates: 1.3–1.5 mm rostral to calamus scriptorius, 1.8–2.1 lateral to the midline, and 2.2–2.6 mm ventral to the dorsal surface of the brain stem. Baro-activated CVLM neurons were identified by five criteria: 1) spontaneous activity when arterial baroreceptors were active (i.e., when lowering AP with nitroprusside increased SNA), 2) discharge rate briskly increased by slightly raising AP (≥4 times above baseline), 3) discharge rate strongly modulated by the AP pulse, 4) lack of obvious respiratory-related activity, and 5) location within the CVLM often immediately ventral to cells with respiratory-related activity. Baro-inhibited CVLM neurons were identified using the same criteria, except they were inhibited by raising AP with constriction of the aortic snare.

We examined the responses of CVLM units to increases and decreases in AP produced by constriction of the aortic snare and nitroprusside (5 μg/kg iv), respectively. Resting barosensitive baroreceptors and nitroprusside were also examined, because GABAergic CVLM neurons have been implicated in the sympathoinhibition elicited by activation of the Bezold-Jarisch reflex with this serotonin 5-HT₄ agonist (Verberne and Guyenet 1992). In addition, to determine whether CVLM neurons could be inhibited by stimuli that elevate presympathetic RVLM neuronal activity and AP, we examined the effects of a brief but firm pinch to the base of the tail (noxious stimulus, Sun and Spyer 1991). The CVLM neurons were not antidromically activated from the RVLM, to preserve the integrity of the tissue for tracing axons of individually filled neurons.

After CVLM neurons were physiologically characterized, they were filled with biotinamide using a previously described juxtacellular labeling method (Pinault 1996; Schreihofer and Guyenet 1997; Schreihofer et al. 1999, 2000). Positive current pulses were delivered through the recording pipette (200-μs pulses of 1.0–4.0 nA at 2.5 Hz for 1–5 min) while the activity of the isolated cell was monitored. The successful entrainment of the cell’s activity to the current pulses produces the label of a single cell in the vast majority of cases (Pinault 1996; Schreihofer and Guyenet 1997). Juxtacellular labeling was limited to one attempt on each side of the medulla.

Physiological data analyses

All physiological variables (AP, HR, end-expired CO₂, SNA, and CVLM unit activity) were monitored on a chart recorder and simultaneously stored on a videocassette recorder via a Vetter interface (frequency range: DC-22 KHz). Subsequent processing was made with a Power 1401 interface and Spike 2 software (version 3, Cambridge Electronic Design, Ltd., www.ced.co.uk). The SNA signal was amplified and filtered (0.20–3 KHz; 60-Hz notch filter) as described previously (Schreihofer and Guyenet 2000; Schreihofer et al. 2000). The extracellular unit signals were amplified and filtered (0.2–3 KHz), and a window discriminator was used to determine neuronal discharge rates.

Histology

At the end of the experiment the rat was deeply anesthetized with halothane and perfused transcardially with PBS (pH 7.4) followed by formaldehyde (4% in 0.1 mM phosphate buffer, pH 7.4). The brain was removed and stored in fixative for 24 h at 4°C. Using a Vibratome (The Vibratome Company, www.vibratome.com) 30-μm coronal sections were cut through the medulla and stored in a cryoprotectant solution (Schreihofer and Guyenet 1997) at −20°C.

Most biotinamide-filled neurons (using 1.5% biotinamide) were processed to identify the phenotype and location of the physiologically characterized neuron. The baro-activated cells were examined for expression of GAD67 mRNA to determine whether they were GABAergic and processed for choline acetyltransferase (ChAT) immunoreactivity to determine whether they could be vagal motor neurons. The baro-inhibited cells, which were not expected to be GABAergic or cholinergic, were examined for GAD67 mRNA as a negative control. In addition, to determine whether baro-inhibited cells were norecholaminergic, they were examined for tyrosine hydroxylase immunoreactivity (Schreihofer and Guyenet 1997; Stornetta et al. 1999; Verberne et al. 1999).

The biotinamide-filled cell was first revealed by incubating the tissue with streptavidin Alexa 448 (1:200 with 0.1% Triton X-100, Molecular Probes) and mounting the sections onto sterile slides in sterile phosphate buffer (pH 7.4). Sterile coverslips were placed onto the sections and the biotinamide-filled somata was located using an epifluorescence microscope. The section containing the cell body was gently removed and processed to detect GAD67 mRNA by in situ hybridization (see next section for details). This section then was further processed to reveal ChAT or tyrosine hydroxylase (TH) immunoreactivity. The adjacent sections were reserved to attempt reconstruction of biotinamide-filled dendrites and axons (in the absence of obscuring hybridization reaction product).

Unless otherwise noted, all incubations and rinses were performed on free-floating sections at room temperature on a shaker in Tris-buffered saline (TBS, pH 7.4). After undergoing in situ hybridization for GAD67 mRNA, the section containing the biotinamide-filled soma was blocked in heat-inactivated horse serum (10%, 30 min, Life Technologies, www.lifetech.com). To reveal ChAT immunoreactivity, the sections were then incubated with a goat polyclonal anti-CHAT primary antibody (1:50, Chemicon, www.chemicon.com) in 0.1% Triton X-100 and 10% horse serum overnight at 4°C, followed by incubation with donkey anti-goat IgG-Cy3 (1:200, 1 h, Jackson ImmunoResearch, www.jacksonimmuno.com). Immunoreactivity for
TH was revealed by incubation with a monoclonal mouse anti-TH antibody (1:1000, Chemicon) followed by incubation with donkey anti-mouse IgG-Cy3 (1:200, 1 h, Jackson). Sections were mounted onto uncoated slides and coverslips were applied with Vectashield (Vector) and affixed with nail polish.

A subset of baro-activated cells was stained strictly for reconstruction of their dendrites and axonal processes. In these cases the phenotype was not determined, but the visualization of the cell was enhanced by increasing the concentration of biotinamide in the recording electrode (5%), a longer survival time (3–4 h), and visualization with a peroxidase-induced reaction product. The sections were incubated in hydrogen peroxide (1%, 30 min) followed by an avidin–biotin solution (2 h, Elite ABC, Vector). Sections were then blocked in normal goat serum (1%, 30 min) followed by a biotinylated goat anti-rabbit secondary antibody (1:400, 1 h), and then a second incubation with an avidin–biotin solution (2 h). The biotinamide-filled cell was revealed by 5- to 10-min incubation with a diaminobenzidine (DAB) solution (0.005% hydrogen peroxide and 0.05% DAB (Sigma Chemicals, www.sigma-aldrich.com). Sections were mounted onto gelatin-coated slides and cleared in graded alcohols and xylenes. Coverslips were applied with DPX (Aldrich, Milwaukee, WI).

**In situ hybridization histochemistry for GAD67 mRNA**

In situ hybridization histochemistry for detection of GAD67 mRNA was performed using antisense digoxigenin-labeled cRNA probes as previously described (Schreihofer et al. 1999; Stornetta and Guyenet 1999). The riboprobes were generated from a full-length cDNA encoding GAD67 (2.7 kb, generously supplied by Dr. A.J. Tobin, University of California, Los Angeles) (Erlander et al. 1991) cloned into pBluescript SK+ (Stratagene). Plasmids were linearized with Sall (Promega, www.promega.com) and transcribed using T3 polymerase (Promega) with digoxigenin-11-UTP (Roche Applied Science, www.biochem.roche.com) as the label. The template DNA was digested with RQ1 DNase (Promega) for 20 min at 37°C. Incorporated nucleotides were removed by Probe Quant G-50 Micro Columns (Amersham Pharmacia Biotech, www.apbiotech.com).

The sections containing the biotinamide-filled soma was rinsed in sterile saline and placed in a prehybridization solution (Schreihofer et al. 1999) at room temperature for 30 min and then at 37°C for 1 h. The riboprobe (50–100 pg/μl) then was added directly to the solution to hybridize for 16 h at 55°C. The sections were washed in 4× SSC with 10 mM sodium thiosulfate (40 min, 37°C) followed by incubation in RNase A solution (1 h, 37°C, 20 μg/ml [in mM] 500 NaCl, 10 Tris–HCl, 1 EDTA, pH 8.0). After rinsing in RNase A buffer (20 min), the sections were washed at increasing stringencies as follows: 2× SSC and 0.5× SSC (20 min each, 37°C) and 0.1× SSC (2 h, 55°C).

The digoxigenin-labeled riboprobe was revealed by incubation with a sheep polyclonal anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1000, overnight, 4°C, Roche) with 10% heat-inactivated normal horse serum (Life Technologies) and 0.1% Triton X-100. The next day the sections were rinsed and incubated in NMT (0.1 M NaCl, 50 mM MgCl2, and 0.1 M Tris, 10 min). The blue–brown reaction product was produced by incubation of the sections in NMT with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (Roche) in the dark (1–3 h) (Schreihofer et al. 1999).

**Mapping and imaging of labeled neurons**

Brightfield illumination was used to visualize some biotinamide-labeled cells (revealed with DAB) and GAD67 mRNA hybridization reaction product. Landmarks within the sections were identified using darkfield illumination. Epifluorescence was used to visualize some biotinamide-labeled neurons (Alexa 488) and ChAT or TH immunoreactivity (Cv3). The location of each biotinamide-filled soma was plotted along with an outline of the section and major landmark structures using a motor-driven stage (Ludl Electronic Products, Hawthorne, NY) and the NeuroLucida system (Microbrightfield, Colchester, VT, www.microbrightfield.com) as previously described (Schreihofer and Guyenet 1997; Stornetta and Guyenet 1999). Some biotinamide-filled neurons were serially reconstructed using the NeuroLucida system.

On a series of representative sections through the CVLM, cells with GAD67 mRNA, TH, and ChAT were plotted to determine the distribution and extent of overlap among the populations of these cell types within the region. The phenotype of each biotinamide-filled neuron was determined, and examples of biotinamide-filled neurons were photographed using a 12-bit color CCD camera (Cool Snap, Roper Scientific, www.roperscientific.com). The resulting tiff files were imported into Adobe Photoshop (6.0, Adobe Systems, www.adobe.com), where they were converted to grayscale with the levels and sharpness adjusted to optimize visualization of the cells.

**RESULTS**

**Physiological properties of baro-activated CVLM neurons**

In 17 rats we recorded from 19 baro-activated neurons (1 rat had 1 recorded neuron on each side of the medulla). Although physiological data presented includes the 19 recorded neurons, 12 were identified phenotypically, 3 were used to examine morphology, and 5 were either not labeled or not found after histological processing. Baseline mean AP was 109 ± 3 mmHg and the basal firing rate of the CVLM neurons was 3.4 ± 0.6 spikes/s. All cells were vigorously activated by slight increases in AP (Fig. 1A) and were further activated as AP rose (4- to 25-fold increase above baseline activity). This excitation was always maintained for the duration of the increased AP (≥1 min, Fig. 1A). In addition, the activity of these baro-activated neurons was strongly modulated by the AP pulse (Fig. 1B). Pulse synchrony was particularly evident at high AP levels (Fig. 1C). Most neurons became silent when AP was lowered below the resting level either by release of the aortic snare (Fig. 1A) or by injection of nitroprusside (13/19 cells, AP to 73 ± 3 mmHg). In contrast, some baro-activated neurons remained active after AP was lowered below baroreceptor threshold by injection of nitroprusside (6/19 cells; AP to 55 ± 4 mmHg; phenotype of 3 of these cells not identified). Three of 6 cells showed no change in activity with nitroprusside, and 3 of 6 showed a decrease in firing rate. The change in AP produced by nitroprusside and the baseline firing rates of cells silenced by decreasing AP (5.2 ± 1.5 spikes/s) were not different from cells that remained active after injection of nitroprusside (3.4 ± 0.6 spikes/s; unpaired t-test, P < 0.05).

Baro-activated CVLM neurons were examined for responses to phenyl biguanide, a 5-HT3 agonist that activates cardiopulmonary afferents to elicit sets of cardiorespiratory responses known as the Bezold–Jarisch reflex. As previously reported (Verberne and Guyenet 1992), intravenous administration of this 5-HT3 agonist (50 μg/kg) produced marked decreases in SNA and AP (Fig. 1D). It has been speculated that the sympathoinhibitory response is mediated by activation of GABAergic CVLM neurons, which inhibit presynaptic RVL neurons (Verberne and Guyenet 1992). As we have previously shown (Verberne et al. 1999), baro-activated CVLM neurons display a burst of activity with injection of phenyl biguanide (Fig. 1D). Accordingly, 17/17 baro-activated CVLM neurons tested were excited by phenyl biguanide, and most of them showed a secondary inhibition of activity as AP decreased (11/17 cells, Fig. 1D). This inhibitory response is
likely due to unloading of the arterial baroreceptors as AP fell. The initial excitatory response to phenyl biguanide appeared to be specific for baro-activated neurons within the CVLM. Random CVLM neurons that showed no barosensitivity or obvious respiratory-related activity were not responsive to phenyl biguanide (8 cells, not shown).

To examine whether baro-activated CVLM neurons decrease their activity during other stimuli that activate SNA, barosensitive CVLM neurons were examined for responses to a noxious stimulus produced by brief tail pinch. This stimulus increases SNA, AP, and the activity of presympathetic RVLM neurons (Sun and Spyer 1991). A brief but firm pinch of the base of the tail produced a reliable burst in SNA followed immediately by a small rise in AP (Fig. 2C). Most baro-activated CVLM neurons (8/11 cells) were clearly inhibited by tail pinch (Fig. 1E). A few baro-activated CVLM neurons (3/11 cells) did not respond to tail pinch, but the phenotype of these neurons was not identified.

**Physiological properties of baro-inhibited CVLM neurons**

In 8 rats we recorded from 10 baro-inhibited CVLM neurons (2 rats had 1 recorded cell on each side of the medulla). Baseline mean AP was 100 ± 4 mmHg, and the basal firing rate of the CVLM neurons was 3.2 ± 1.6 spikes/s. Every cell was totally silenced by increasing AP with the aortic snare, and this silence was maintained throughout the duration of the increased AP (≤1 min; Fig. 2A). The persistence of this inhibitory response to increased AP was mirrored in the recorded SNA (Fig. 2A). Most baro-inhibited CVLM neurons were activated by lowering AP by injection of nitroprusside (6/8 cells; Fig. 2A).

Every baro-inhibited CVLM neuron tested with phenyl biguanide was inhibited (9 cells; Fig. 2B). Most baro-inhibited CVLM neurons (6/9 cells) displayed a secondary excitatory response to phenyl biguanide as AP fell and SNA increased (Fig. 2B). Most baro-inhibited CVLM neurons (3/4 cells) displayed a brief burst in activity during the pinch (Fig. 2C), followed by an inhibition as AP increased.

**Location and phenotypes of barosensitive CVLM neurons**

Twelve baro-activated CVLM neurons were successfully labeled with biotinamide and later found during histological processing. All 12 cells were located where expected within...
the CVLM, i.e., approximately 1.3–1.5 mm caudal to the facial nucleus near bregma level –13.0 mm (Paxinos and Watson 1998). Three of these cells were examined after sectioning the brain in the saggital plane to determine whether this would facilitate reconstruction of processes. However, it quickly became apparent that most of the dendrites and the initial course of the axon occurred within the coronal plane (Schreihofer and Guyenet 2002), and most biotinamide-filled neurons were examined in the coronal plane (9/12 cells). The location of each soma was found reliably between the rostral wings of the lateral reticular nucleus (Fig. 3A). These cells were located 626 ± 36 μm from the ventral surface and 1.64 ± 0.03 mm lateral to the midline. Seven baro-inhibited neurons were also filled with biotinamide and their locations (525 ± 55 μm from the ventral surface and 1.72 ± 0.04 mm lateral to the midline) were not different from that of the baro-activated CVLM neurons (Fig. 3B; unpaired t-test, P < 0.05).

The CVLM contains at least two types of neurons that are likely to be nonrespiratory and activated by increased AP, namely GABAergic interneurons and cholinergic cardiovagal motor neurons. Here we sought to determine whether baro-activated CVLM neurons with strong pulse-modulated activity are indeed GABAergic by demonstrating the presence of GAD67 mRNA and absence of ChAT immunoreactivity. In addition, we examined whether there is any overlap between populations of cholinergic neurons with GABAergic neurons within the CVLM. We have previously shown that some CVLM neurons inhibited by increased AP are catecholaminergic (Verberne et al. 1999) and that catecholaminergic CVLM neurons are not GABAergic (Stornetta and Guyenet 1999). Here we sought to demonstrate that baro-inhibited CVLM neurons are not GABAergic and to confirm their catecholaminergic phenotype. We also examined whether cholinergic and catecholaminergic neurons are distinct populations of neurons within the CVLM.

The CVLM contained a high density of neurons with GAD67 mRNA except in the nucleus ambiguus and lateral reticular nuclei (Fig. 4A; Table 1). Large ChAT-immunoreactive neurons were concentrated within the nucleus ambiguus (Figs. 4B, 5A1, and 5B1), which never displayed GAD67 mRNA (Fig. 5A2). Smaller ChAT-immunoreactive neurons were also found throughout the CVLM (Figs. 4B and 5B4), and the vast majority of these neurons displayed no GAD67 mRNA (Figs. 4B and 5B2, Table 1). However, a few cholinergic neurons near the ventral surface of the brain stem also contained GAD67 mRNA (Figs. 4B and 5C, Table 1). These neurons were ventral to the region containing the recorded CVLM neurons (Fig. 3). The TH-immunoreactive neurons were interspersed throughout the CVLM but were distinct from cholinergic neurons (Fig. 4B, Table 1) and GABAergic neurons (Figs. 4 and 5D, Table 1).

The 12 biotinamide-filled baro-activated CVLM neurons were examined for GAD67 mRNA. Most of these neurons (9/12 cells) contained intense hybridization reaction product for GAD67 mRNA within their small cytoplasms (Figs. 6, A and B). Five of the nine cells were also examined for ChAT immunoreactivity, which was absent (Fig. 6A3 and B3), although nearby large neurons within nucleus ambiguus dis-
played strong immunoreactivity for ChAT (Fig. 6B). The biotinamide-filled cells were dorsal to the few cholinergic neurons with GAD67 mRNA. The other 3 biotinamide-filled cells displayed a weak hybridization signal for GAD67 mRNA (ambiguous labeling), but none of these neurons were ChAT immunoreactive.

Seven of the baro-inhibited CVLM neurons were filled with biotinamide, and all of them were found to be TH immunoreactive (Fig. 6C). Although TH immunoreactivity alone predicts an absence of GAD67 mRNA (Stornetta and Guyenet 1999), five of these neurons were also examined for GAD67 mRNA, which was clearly absent in every case (Fig. 6C). Thus the baro-inhibited cells that were examined histologically were catecholaminergic, and none of them were GABAergic.

**Structures of baro-activated CVLM neurons**

For cells labeled using 1.5% biotinamide, the section containing the soma was processed to determine phenotype, and the adjacent sections were used to serially reconstruct dendritic and axonal processes. Although limited reconstruction was possible using this method (for 6 cells), the distance in which the putative axon could be followed was 500 μm at most, and many cells could not be traced beyond a few adjacent sections. The dendrites were usually smooth, with little branching and trajectories in all directions mostly within the coronal plane (within 300 μm rostrocaudally). The lateral extension of the dendrites was limited by the lateral reticular nuclei, and the processes ventured predominantly dorsally and ventrally. The putative axons exited the soma dorsomedially and projected toward the dorsolateral tegmentum and rostrally. No putative axons were observed projecting caudally from the soma.

To enhance reconstruction, three baro-activated CVLM neurons were labeled with 5% biotinamide and revealed with nickel-DAB. Although this process did not allow for phenotypic identification of the neuron, it facilitated reconstruction of the putative axon. Two cases are illustrated along with their physiological characterizations in Figs. 7 and 8. In the first case, the CVLM neuron was briskly activated by increased AP.
which was sustained for 1 min (Fig. 7). The neuron was also inhibited by decreasing AP with nitroprusside (Fig. 7). In addition, this neuron was excited by phenyl biguanide, but showed no response to tail pinch (not shown). Reconstruction showed the usual smooth, sparsely branching dendrites and a putative axon that coursed dorsomedially toward the dorsal tegmentum. This process had multiple discernible branches medially within the CVLM and at the level of the nucleus ambiguus. In addition, as the putative axon rose to the dorsal half of the section, branches were observed both laterally and at the midline. The axon projected rostrally along its dorsal course to produce two branches that coursed dorsomedially toward the dorsal tegmentum. This neuron showed a similar axonal projection pattern, with axonal branches dorsal to the soma and then gave rise to a projection pattern definitive of a vagal motor neuron. The axon ascended dorsomedially without further branching and looped around to descend ventrolaterally and parallel to the ascending limb of axon. The descending axonal fiber exited the ventrolateral medulla. Although the phenotype of this neuron was not determined, its axonal projection is typical of a cholinergic vagal motor neuron.

DISCUSSION

The present study provides the first demonstration of the GABAergic phenotype of individually recorded CVLM neurons that respond to changes in AP, stimulation of the Bezold–Jarisch reflex, and noxious tail pinch. In particular, these data supply conclusive evidence that the CVLM neurons with the electrophysiological properties consistent with a baroreceptor reflex role are indeed GABAergic.

We have previously used the powerful combination of extracellular recording, juxtacellular labeling, and immunohistochemistry of individual neurons to conclusively identify the phenotypes and structures of presympathetic RVLM neurons (Schreibhofer and Guyenet 1997). The present study took advantage of this approach combined with in situ hybridization for GAD67 mRNA (Schreibhofer et al. 1999) to determine whether neurons in the CVLM that displayed the physiological characteristics of baroreceptor reflex interneurons are GABAergic. The central circuitry for the baroreflex, which is drawn with a GABAergic interneuron in CVLM that receives excitatory baroreceptor-related inputs via the NTS and relays them to the RVLM (Chan and Sawchenko 1998; Sved and Gordon 1994), is based on a variety of suggestive evidence. The CVLM contains neurons that are innervated by the NTS, which in turn project to the CVLM (Aicher et al. 1995). In addition, the CVLM contains GABAergic neurons that project to the RVLM and express Fos with sustained increases in AP, suggesting they are excited by increased baroreceptor inputs (Chan and Sawchenko 1998; Minson et al. 1997). Extracellular recordings of neurons within the CVLM demonstrate neurons that are activated by small increases in AP, show pulse-modulated activity, and project toward the RVLM (Agarwal and Calaresu 1991; Gieroba et al. 1992; Jeske et al. 1993; Terui et al. 1990). However, these studies did not identify the phenotype of the recorded neurons. The present study fills this critical gap in our knowledge by demonstrating that the neurons with the appropriate physiological characteristics are indeed GABAergic.

The physiological responses of this cell were indistinguishable from other baro-activated CVLM neurons, its reconstruction produced strikingly different results. The dendrites were varicose with branches extending only ventrally. The axon projected dorsomedially with local branches immediately dorsal to the soma and then gave rise to a projection pattern definitive of a vagal motor neuron. The axon ascended dorsomedially without further branching and looped around to descend ventrolaterally and parallel to the ascending limb of axon. The descending axonal fiber exited the ventrolateral medulla. Although the phenotype of this neuron was not determined, its axonal projection is typical of a cholinergic vagal motor neuron.

Projections of baro-activated CVLM neurons

Although GABAergic CVLM neurons are often depicted as a simple relay to the RVLM, these cells are likely to innervate...
multiple targets to provide a more widespread inhibition of the CNS by baroreceptors and other inputs. Although we could not demonstrate the points of termination of the axons from cells reconstructed in the present study, there were obvious branches, as the axon coursed dorsally and then rostrally. The observation that the axon crosses to the other side of the medulla is in agreement with the notion that CVLM neurons on each side innervate both sides of the RVLM. Baro-activated CVLM neurons have been antidromically activated from the ipsilateral and contralateral RVLM regions (Gieroba et al. 1992). In addition, activation of the CVLM can inhibit presym pathetic RVLM neurons on both sides of the medulla (Li et al. 1991; Masuda et al. 1991). Although neither antidromic activation nor partial reconstruction of axons conclusively dem-

**FIG. 6.** Examples of biotinamide-filled CVLM neurons with GAD67 mRNA, ChAT immunoreactivity, and TH immunoreactivity. Baro-activated CVLM neurons filled with biotinamide and revealed with streptavidin Alexa 488 (A1 and B1) displayed intense hybridization reaction product for GAD67 mRNA (A2 and B2). These neurons were not ChAT-ir (A1 and B1) but were near large, putative motor neurons that were ChAT-ir (B3). C1: a biotinamide-filled, baro-inhibited CVLM neuron that had no detectable GAD67 mRNA (C2) but was TH-ir (C3). Bars, 25 μm.
Demonstrate a direct connection to presympathetic RVLM neurons, several observations strongly suggest at least some if not most of these CVLM neurons are likely to provide a direct inhibition. Extracellular recordings of GABAergic CVLM neurons in the present study demonstrate that their activity has an inverse relationship to SNA and reported activities of presympathetic RVLM neurons (Brown and Guyenet 1985; Morrison et al. 1988). Furthermore, stimulation of the CVLM inhibits presympathetic RVLM neurons, SNA, and AP (Blessing 1988; Masuda et al. 1991) via activation of GABAergic receptors in the RVLM (Blessing 1988). Thus many of the recorded neurons in the present study are likely to provide a baroreflex link between the NTS and the RVLM. However, these cells are also likely to play a role in baroreceptor-mediated inhibition of other regions of the CNS.

Phenotypic identification of barosensitive CVLM neurons

Most baro-activated CVLM neurons labeled with biotinamide in this study were GABAergic and not vagal motor neurons, as indicated by a lack of ChAT immunoreactivity and their axonal projections. However, one reconstructed baro-activated CVLM neuron clearly had the axonal projection typical of a vagal motor neuron. In addition, it is possible that some of the baro-activated CVLM neurons not filled with biotinamide (5/17) also could have been cardiovagal motor neurons. Interestingly, the reconstructed motor neuron also displayed local axon collaterals at the level of the nucleus ambiguus (Fig. 8). This neuron was ventral to the dense concentration of cholinergic neurons of the nucleus ambiguus, but we also observed cholinergic neurons intermingled with GABAergic neurons throughout the CVLM. Remarkably, the

FIG. 7. Example of a baro-activated CVLM neuron that was physiologically characterized and later reconstructed. Top left: constriction of the aortic snare (bar above AP trace) raised AP and activated the CVLM neuron for 1 min. These responses were accompanied by sustained decreases in HR and splanchnic SNA. Top right: injection of nitroprusside (arrow) decreased AP and inhibited the CVLM neuron. These responses were accompanied by increases in HR and SNA. Bottom left: reconstruction of the recorded neuron within the representative coronal section shows the soma within the CVLM between the lateral wings of the lateral reticular nucleus. Arrowheads indicate branch points of the putative axon. Bottom right: magnified view of the same reconstructed neuron to highlight small size of soma and potential axon collaterals.
physiological responses of this neuron were indistinguishable from the identified GABAergic CVLM neurons with the tests employed in the present study, highlighting the importance of phenotypic identification or verification of rostral axonal projection of individually recorded neurons.

Among the baro-activated CVLM neurons we encountered neurons that were silenced by increasing AP. In the present study these neurons served as a negative control for detection of GAD67 mRNA in neighboring baro-activated neurons. These baro-inhibited CVLM neurons may be caudal C1 neurons that project to the forebrain as described previously (Stornetta et al. 1999; Verberne et al. 1999), and there is no overlap between the populations of GABAergic and catecholaminergic neurons within the brain stem (Stornetta and Guyenet 1999).

As expected, none of the baro-inhibited CVLM neurons expressed GAD67 mRNA but were instead immunoreactive for TH. The responses of these neurons resemble those of the presympathetic RVLM neurons, which are positively correlated with SNA. They were silenced by activation of baroreceptor inputs or cardiopulmonary receptors with phenylbiguanide. In addition, most of them were activated by firm tail pinch, as previously described for hypothalamic projecting C1 cells (Verberne et al. 1999) and bulbospinal C1 cells (Sun and Spyer 1991; Verberne et al. 1999). The source of their inhibition by baroreceptor and cardiopulmonary stimuli is not known at present, as no discernible local terminals were observed in our baro-activated CVLM neurons. However, some of our baro-activated neurons did give rise to potential local axonal

FIG. 8. Example of a baro-activated CVLM neuron that was physiologically characterized and later reconstructed. Top left: constrictions of the aortic snare (bars above AP trace) raised AP and activated the CVLM neuron. These responses were accompanied by sustained decreases in HR and splanchnic SNA. Top right: injection of nitroprusside (arrow) decreased AP and inhibited the CVLM neuron. These responses were accompanied by increases in HR and SNA. Bottom right: reconstruction of the recorded neuron within the representative coronal section shows the soma within the CVLM between the lateral wings of the lateral reticular nucleus. Arrowheads indicate branch points of the putative axon with the trajectory typical of a vagal motor neuron. Bottom left: magnified view of the same reconstructed neuron to highlight convoluted dendritic paths and potential axon collaterals.
branches that could be a possible source of inhibition to neurons in the ventral medulla (Fig. 7).

**Encoding of AP by neurons in the baroreflex pathway**

Baroreceptor afferents projecting to the NTS exhibit a sigmoidal relationship to mean AP (Chapleau and Abboud 1987; Jones and Thoren 1977; Seagard et al. 1990), which is preserved but inversely related to the outputs of the system, SNA and HR. In addition, this inverse relationship is reflected in the activity of presympathetic RVLM neurons (Brown and Guyenet 1985). The baro-activated GABAergic CVLM neurons of the present study also appeared to faithfully encode the mean level of AP. The activity of these neurons increased as AP was raised by constricting an abdominal aortic snare, and this level of AP. The activity of these neurons increased as AP was raised by constricting an abdominal aortic snare, and this activation was sustained with no apparent accommodation raised by constricting an abdominal aortic snare, and this level of AP. The activity of these neurons increased as AP was raised by constricting an abdominal aortic snare, and this activation was sustained with no apparent accommodation throughout the duration of a steady elevated AP for ≥1 min (Figs. 1 and 7). Brief fluctuations in AP during this hypertensive period were also markedly reproduced by changes in the discharge rate of the CVLM neurons (Fig. 1). These data agree with previous observations of positive correlations between the discharge rates of baro-activated CVLM neurons and mean AP (Jeske et al. 1993). This was also true of the baro-activated vagal motor neuron, where a sustained and stable increase in AP was mirrored in the persistent activation of the neuron (Fig. 8) and sustained bradycardia, consistent with previous findings (Gieroba et al. 1992). In contrast, another report observed activations of CVLM neurons that were not sustained during the elevation of AP, possibly activated by a change in AP and then decreasing activity as AP reached a new plateau (Gieroba et al. 1992). However, the phenotypes of the neurons in this study were not identified. Nevertheless, increased AP produces a sustained inhibition of SNA (see Figs. 2, 7, and 8), which is likely to be produced by the persistent inhibition of presympathetic RVLM neurons by the CVLM. The pattern of activity of the baro-activated CVLM neurons recorded in the present study is consistent with their potential role in baroreceptor-mediated changes in SNA.

In contrast to the behavior observed in GABAergic CVLM neurons, most baro-activated NTS neurons are described as briskly responsive to changes in mean AP, but display rapidly adaptive responses to sustained increases in AP (Mifflin 2001; Rogers et al. 1996; Seagard et al. 1995; Zhang and Mifflin 2000). Only a minority of the baro-activated NTS neurons exhibit a nonadapting increase in activity in response to a rise in AP (Seagard et al. 1990). Conceivably, the latter may be higher-order neurons in the baroreflex pathway. Although there may be second-order NTS neurons that do encode the mean level of AP, it is also possible that some level of processing occurs within the NTS before the baroreceptor-related information is relayed to the CVLM.

Further evidence that the baro-activated CVLM neurons described in the present study may be part of the baroreceptor reflex pathway is their strong modulation of activity by the AP pulse. All of the baro-activated CVLM neurons in the present study were strongly activated immediately following the peak in AP at early diastole, as previously reported (Jeske et al. 1993). In fact, when AP was raised, the discharge of the neurons became pulse synchronous (Fig. 1C). Although baroreceptor afferents display pulse-modulated activity (Brown 1980), reports in NTS neurons have inconsistently seen this behavior (Rogers et al., 1993, 1996; Seagard et al. 1995). Nevertheless, modulation of GABAergic CVLM neuronal activity by the AP pulse likely contributes to the strong pulse modulation of discharge rate observed in presympathetic RVLM neurons and SNA (Guyenet and Brown 1986; Morrison et al. 1988).

**Activation of GABAergic CVLM neurons by the Bezold–Jarisch reflex**

In addition to their role in relaying arterial baroreceptor-related information, GABAergic CVLM neurons have been proposed to mediate the sympathoinhibitory response of the Bezold–Jarisch reflex (Verberne and Guyenet 1992), which is elicited by stimulation of 5-HT3 receptors on chemosensitive cardiac receptors with vagal afferents that project to the NTS. Intravenous injection of phenyl biguanide, a 5-HT3 receptor agonist, elicits robust decreases in SNA and AP (Figs. 1D and 2B), which are most likely produced by inhibition of presympathetic RVLM neurons (Verberne and Guyenet 1992). Phenyl biguanide injection reduces the firing rates of presympathetic RVLM neurons, and blockade of GABAergic receptors in the RVLM reduces the sympathoinhibition and hypotension normally observed with injection of phenyl biguanide (Verberne and Guyenet 1992). Our laboratory has previously reported the stimulation of baro-activated CVLM neurons by phenyl biguanide (Verberne et al. 1999), although the phenotype of these cells was not identified. The present study provides the first demonstration that GABAergic CVLM neurons are activated by phenyl biguanide, suggesting that these cells may contribute to the GABAergic inhibition of presympathetic RVLM neurons by production of the Bezold–Jarisch reflex.

**Integration of spinal signals by GABAergic CVLM neurons**

The CVLM is most recognized as a region that relays cardiopulmonary information from the NTS to presympathetic RVLM neurons to modulate SNA and AP. However, inputs from the spinal cord arising from somatic, visceral, and nociceptive afferents, which modulate presympathetic RVLM activity (Ermiro et al. 1993; Masuda et al. 1992; Peng et al. 2002; Sun and Spyer 1991), also appear to be integrated through the CVLM. For example, stimulation of greater splanchnic afferents stimulates baro-activated CVLM neurons and lowers AP, and blockade of glutamate receptors in the CVLM markedly attenuates this depressor response (Peng et al. 2002). Likewise, stimulation of sural nerve produces a depressor response that is attenuated by blockade of GABAergic receptors in the RVLM or inhibition of the CVLM with kainic acid (Masuda et al. 1992). In the present study we examined whether a noxious stimulus delivered by firm tail pinch modulated the activity of GABAergic CVLM neurons. Most baro-activated CVLM neurons were indeed inhibited by a tail pinch that also produced increases in SNA and AP, suggesting that a reduction in their activity could contribute to the activation of presympathetic RVLM neurons. Although the withdrawal of baroreceptor inputs can lower the activity of GABAergic CVLM neurons, these data are the first demonstration of an evoked stimulus that inhibits the activity of GABAergic CVLM neurons.
Baroreceptor-independent activation of GABAergic CVLM neurons

In addition to its established role in baroreceptor-mediated inhibition of presympathetic RVLM neurons and SNA, the CVLM also provides tonic baroreceptor-independent inhibition to presympathetic RVLM neurons (Cravo and Morrison 1993; Guyenet et al. 1987). Whereas most of the baro-activated neurons in the present study were silenced by decreasing AP with nitroprusside or release of the aortic snare, some of these GABAergic CVLM neurons remained active after AP had been lowered below baroreceptor threshold. These data indicate that some GABAergic CVLM neurons with apparent cardiovascular function are driven by other excitatory inputs. Clearly, the baroreceptors via the NTS provide a tonic glutamatergic input to the CVLM (Gordon 1987; Guyenet et al. 1987). However, the baroreceptor-mediated drive to CVLM appears to be only a part of the glutamatergic influence to these cells (Guyenet et al. 1987). The source of this excitation may be derived in part from the NTS, given that many baro-activated NTS neurons remain active after baroreceptors have been unloaded (Zhang and Miftlin 2000). However, the CVLM appears to tonically inhibit the RVLVM even in the absence of the NTS (Dampney et al. 1988; Schreihoffer et al. 1996), suggesting the existence of other sources of tonic excitatory inputs to the CVLM. The present study suggests that some CVLM neurons may process both baroreceptor-related and baroreceptor-independent inputs.

In conclusion, the CVLM is known to play an important role in the regulation of SNA by providing the major tonic inhibitory influence to presympathetic RVLM neurons. The present study identifies GABAergic CVLM neurons that are driven by baroreceptor inputs and modulated by the pulse of AP to potentially influence SNA via the baroreflex. These same GABAergic CVLM neurons appear to process excitatory and inhibitory inputs from other sources and project to a number of targets, suggesting that GABAergic CVLM neurons integrate multiple sensory inputs from the brain stem and spinal cord to provide a widespread inhibitory influence on autonomic function.

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