Role of presympathetic C1 neurons in the sympatholytic and hypotensive effects of clonidine in rats

ANN M. SCHREIHOFER AND PATRICE G. GUYENET

Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908-0735

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Schreihofor, Ann M., and Patrice G. Guyenet. Role of presympathetic C1 neurons in the sympatholytic and hypotensive effects of clonidine in rats. Am J Physiol Regulatory Integrative Comp Physiol 279: R1753–R1762, 2000.—The rostral ventrolateral medulla (RVLM) may play an important role in the sympatholytic and hypotensive effects of clonidine. The present study examined which type of presympathetic RVLM neurons are inhibited by clonidine. In chloralose-anaesthetized and ventilated rats, clonidine (10 µg/kg iv) decreased arterial pressure (116 ± 6 to 84 ± 2 mmHg) and splanchnic nerve activity (93 ± 3% from baseline). Extracellular recording and juxtacellular labeling of barosensitive bulbospinal RVLM neurons revealed that most cells were inhibited by clonidine (26/28) regardless of phenotype [tyrosine hydroxylase (TH)-immunoreactive cells: 48 ± 7%; non-TH-immunoreactive cells: 42 ± 5%], although the inhibition of most neurons was modest compared with the observed sympathoinhibition. Depletion of most bulbospinal catecholaminergic neurons, including 76 ± 5% of the rostral C1 cells, by microinjection of saporin anti-dopamine β-hydroxylase into the thoracic spinal cord (levels T2 and T4, 42 ng·200 nl−1·side−1) did not alter the sympatholytic or hypotensive effects of clonidine. These data show that although clonidine inhibits presympathetic C1 neurons, bulbospinal catecholaminergic neurons do not appear to be essential for the sympatholytic and hypotensive effects of systemically administered clonidine. Instead, the sympatholytic effect of clonidine is likely the result of a combination of effects on multiple cell types both within and outside the RVLM.

rostral ventrolateral medulla; anti-dopamine β-hydroxylase; saporin; splanchnic nerve activity; tyrosine hydroxylase; phenylethanolamine-N-methyl transferase

CLONIDINE, an α₂-adrenergic receptor agonist, produces a long-lasting decrease in arterial pressure (AP) by a centrally mediated inhibition of sympathetic vasomotor tone. Although multiple sites within the central nervous system are sensitive to clonidine (10, 27), the rostral ventrolateral medulla (RVLM) appears to be an important target for clonidine-induced hypotension (7, 24–26). The decrease in AP produced by intravenously administered clonidine is mimicked by microinjection directly into the RVLM and is reversed by microinjection of the α₂-adrenergic receptor antagonist idazoxan into the same area (24).

Some barosensitive bulbospinal (presympathetic) neurons in the RVLM are inhibited by clonidine administered intravenously and by local microiontophoresis (3, 9, 33). Moreover, the inhibition of RVLM neurons by intravenous clonidine can be attenuated by microiontophoresis of idazoxan into the vicinity of the recorded RVLM neuron (3). The presympathetic RVLM neurons most clearly inhibited by clonidine have relatively low rates of discharge and slow axonal conduction velocities (3, 9, 32, 33), suggesting that they include C1 cells (28). However, the RVLM also contains non-C1 cells that are likely to be important for the generation of sympathetic vasomotor tone (15, 28), and whether these neurons are also inhibited by clonidine is not known.

Several lines of evidence suggest that clonidine may not preferentially target C1 cells in the RVLM. Although bulbospinal C1 cells have postsynaptic α₂-adrenergic receptors (8), which likely contribute to the clonidine-induced inhibition of these cells, clonidine also affects neuronal activity via presynaptic α₂-adrenergic receptors (10, 35). In fact, in the RVLM, the majority of α₂-adrenergic receptors are located in axons and axon terminals (18). Some of these terminals have synaptic contacts with C1 neurons, but contacts are also made with noncatecholaminergic neurons within the RVLM. Furthermore, in neonate rat brain stem slices, stimulation of α₂-adrenergic receptors inhibits bulbospinal RVLM neurons primarily by a presynaptic inhibition of glutamatergic inputs, which is equally effective in catecholaminergic and noncatecholaminergic RVLM neurons (11). However, whether these noncatecholaminergic RVLM neurons recorded in vitro are related to cardiovascular function could not be ascertained.

One goal of the present study was to seek definitive evidence that sympatholytic doses of clonidine inhibit presympathetic C1 neurons. In addition, we sought to determine whether noncatecholaminergic presympathetic RVLM neurons are also inhibited by clonidine. Finally, we tested whether C1 cells are important for the sympatholytic and hypotensive effects of clonidine.
by determining the consequences of destroying the C1 presympathetic neurons with the use of the immunotoxin anti-dopamine β-hydroxylase-saporin (anti-DβH-Sap).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, weighing 250–350 g (Hilltop Laboratories, Scottsdale, PA), were housed in groups (4 rats/cage) with a 12:12-h light-dark cycle wherein food and water were available ad libitum. All procedures were performed in accordance with National Institutes of Health and University of Virginia Animal Care and Use Guidelines.

Surgical procedures and experimental protocol. Anesthesia was induced with 5% halothane (in 100% O₂). Rats were intubated and artificially ventilated with 1.5–1.9% halothane in 100% O₂ during surgical procedures. A brachial artery was cannulated to measure AP and heart rate (HR), and a brachial vein was cannulated to administer anesthetic and paralytic agents. A femoral vein was cannulated for administration of clonidine. An inflatable snare was placed around the abdominal aorta just below the diaphragm to permit rapid control of upper body AP (28). After placing the rat in a stereotaxic apparatus, the left splanchic nerve was isolated as previously described (29) and placed on two Teflon-coated silver wires (250-μm tip bared; A-M Systems, Everett, WA). The wires were embedded in a dental impression material (polyvinylsiloxane; Darby Dental Supply, Everett, WA). The wires were then connected to wires. A laminectomy was performed at spinal segment T2 with a No. 11 scalpel blade. The dura mater was removed and the spinal cord was exposed. The cord was then moved to the left side of the rat, and a 6-0 silk suture was tied around the cord to keep it in place. The Wistar rats were paralyzed with pancuronium bromide (1 mg/kg iv; Elkins-Sinn, Cherry Hill, NJ) to allow for antidromic activation of RVLM neurons.

Presympathetic RVLM neurons were recorded extracellularly with the use of glass electrodes filled with 1.5% biotinamide in 0.5 M NaCl as previously described (28, 36). Units were selected on the basis of the following criteria: 1) location (rostrocaudally within 500 μm of the caudal pole of the facial nucleus, ventrally within 300 μm of the bottom of the facial nucleus, and 1.7–1.9 mm lateral to the midline), 2) spontaneous activity, 3) time-locked inhibition by increased AP, and 4) antidromic activation from the thoracic spinal cord. After characterization of a stable presympathetic RVLM unit, the effects of clonidine were examined.

Baseline parameters were measured for 45 s to 1 min (Fig. 1), and then clonidine was administered in three doses (1, 1.5, and 7.5 μg/kg iv) given at 5-min intervals. Measurements were taken during the minute preceding each dose and at ~10 min after the last dose (Fig. 1). In a subset of animals, clonidine was administered in a single 10-μg/kg dose.

Microinjections of saporin conjugates into the thoracic spinal cord. Anesthesia was induced with 5% halothane, and during surgery rats were maintained with 1.9% halothane inhaled through a nose cone. The rat was placed in a stereotaxic apparatus, and a dorsal laminectomy was performed to expose the thoracic spinal cord. As previously described (29), bulbospinal catecholaminergic neurons were depleted with the use of the ribosomal toxin saporin conjugated to an antibody for dopamine β-hydroxylase (anti-DβH-Sap; Chemicon). Rats received bilateral microinjections of anti-DβH-Sap (42 ng-200 nl⁻¹·injection⁻¹) into the region of the intermediolateral cell column of two levels of the spinal cord (at T2 and T4). Another group of rats received bilateral microinjections of saporin conjugated to a mouse IgG (IgG-Sap; 40 ng-200 nl⁻¹·injection⁻¹; Chemicon) into the same levels of the thoracic spinal cord. Because IgG-Sap does not alter the number of catecholaminergic neurons, these rats served as an operated control group. Rats were allowed to

Biotinamide-labeled neurons were revealed by incubating the sections in streptavidin-Cy3 conjugate (1:1,000; 3 h; Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were mounted onto gelatin-coated slides with coverslips impregnated with the use of a glycerol-based mounting medium (Vectorshield; Vector Laboratories, Burlingame, CA) and examined with the use of a fluorescence microscope. As previously described (28), the biotinamide-labeled cell was located, photographed (standard 35-mm camera and 1600 ASA color slide film), and its structure along with the outline of the section was drawn with the use of a Neurolucida software (Microbrightfield, Colchester, VT) and a Ludl motor driven stage. The sections containing the labeled cell bodies were removed from the slides and processed to reveal tyrosine hydroxylase (TH) immunoreactivity with the use of a sensitive peroxidase-antiperoxidase method (28). Sections were incubated with a mouse monoclonal TH antibody (1:2,000; 24 h at 4°C; Chemicon, Temecula, CA) in 10% blocking serum and 0.1% Triton X-100, followed by a goat-anti-mouse IgG (1:150; 45 min; Sternberger Monoclonal, Baltimore, MD) and then mouse ClonOPAP (1:150; 30 min; Sternberger). Immunoreactivity for TH was revealed by incubation for 5–10 min in a 0.05% 3,3’-diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide solution. Sections were mounted onto gelatin-coated slides, cleared in graded alcohols and xylenes, and coverslipped with DPX (Aldrich, Milwaukee, WI). As previously described (28), to determine whether the recorded neuron was catecholaminergic, the Neurolucida drawing of the biotinamide-labeled cell was superimposed onto the binocular of the microscope with the use of the Lucivid camera system (Microbrightfield). The TH-immunoreactive (TH-ir) neurons were photographed with the use of a 35-mm camera and TMAX-100 black and white film.

Microinjections of saporin conjugates into the thoracic spinal cord. Anesthesia was induced with 5% halothane, and during surgery rats were maintained with 1.9% halothane inhaled through a nose cone. The rat was placed in a stereotaxic apparatus, and a dorsal laminectomy was performed to expose the thoracic spinal cord. As previously described (29), bulbospinal catecholaminergic neurons were depleted with the use of the ribosomal toxin saporin conjugated to an antibody for dopamine β-hydroxylase (anti-DβH-Sap; Chemicon). Rats received bilateral microinjections of anti-DβH-Sap (42 ng-200 nl⁻¹·injection⁻¹) into the region of the intermediolateral cell column of two levels of the spinal cord (at T2 and T4). Another group of rats received bilateral microinjections of saporin conjugated to a mouse IgG (IgG-Sap; 40 ng-200 nl⁻¹·injection⁻¹; Chemicon) into the same levels of the thoracic spinal cord. Because IgG-Sap does not alter the number of catecholaminergic neurons, these rats served as an operated control group. Rats were allowed to
recover 3–5 wk and then were prepared as described above for measuring the effects of clonidine on AP, HR, and splanchnic nerve activity (SNA). No RVLM units were recorded in these animals.

**Verification of lesions by anti-DβH-Sap.** To determine the extent of depletion of bulbospinal catecholaminergic neurons in rats treated with anti-DβH-Sap, brain stem sections from rats treated with anti-DβH-Sap, rats treated with IgG-Sap, and untreated control rats were processed to reveal immunoreactivity for phenylethanolamine-N-methyl transferase (PNMT) and TH. All immunohistochemical procedures were performed with the use of Tris-buffered saline (0.1 M Tris, pH 7.4) at room temperature unless otherwise noted. To determine the loss of C1 neurons in the RVLM, for each animal a one in six series of sections were incubated with a rabbit polyclonal antibody for PNMT (1:2,000 with 10% serum and 0.1% Triton X-100, overnight at 4°C; DiaSorin, Stillwater, MN) followed by a biotinylated goat anti-rabbit IgG (1:200; 45 min; Vector Laboratories). The PNMT-immunoreactive (PNMT-ir) neurons were revealed by incubation with streptavidin-Cy3 conjugate (1:1,000; 1 h; Jackson Immunoresearch Laboratories). To determine the loss of A5 cells in the ventrolateral pons, a separate set of one in six sections were incubated with a mouse monoclonal antibody for TH (1:2,000 with 10% serum and 0.1% Triton X-100 overnight at 4°C; Chemicon) followed by a biotinylated goat anti-mouse IgG (1:200; 45 min; Vector Laboratories). The TH-ir cells were revealed by incubation with streptavidin-Cy3 conjugate (1:1,000; 1 h; Jackson Immunoresearch Laboratories). All sections were mounted onto slides, and coverslips were applied with Krystalon mounting medium (EM Diagnostic Systems, Gibbstown, NJ).

The locations of Cy3-positive neurons were plotted with the use of Neurolucida software and a Ludl motor driven stage as previously described (29). Neuroanatomical nomenclature and planes of sections are derived from Paxinos and Watson (22), with anterior-posterior (A-P) levels in millimeter distance from bregma. To estimate the depletion of bulbospinal C1 cells, rostral PNMT-ir neurons were plotted in sections corresponding to A-P levels −11.8 and −11.6 (2 sections/animal). This method reliably estimates the depletion of bulbospinal neurons, but it may underestimate the depletion of spinally projecting neurons on average by 12% due to interspersed C1 cells that project to the hypothalamus and not to the spinal cord (29, 31). To demonstrate consistency of PNMT immunoreactivity among the groups of rats, PNMT-ir cells were plotted from sections corresponding to A-P levels −13.2 and −13.0. These caudal C1 cells do not project to the spinal cord (28, 31), and they would not be expected to be affected by intraspinal injections of anti-DβH-Sap (29). To determine the depletion of A5 noradrenergic neurons, TH-ir cells were plotted in the ventral half of sections ranging from A-P levels −9.6, −9.4, −9.2, and −9.0 (4 sections/animal).

**Data analyses and statistics.** All physiological measures were monitored on a chart recorder and stored with the use of a video cassette recorder with a digitizer interface (Vetter 3000A, frequency range: DC-22 kHz, Vetter Digital, Rebersberg, VA). Mean AP (MAP), HR, integrated SNA, and integrated rate histograms of RVLM single-unit activity were analyzed with the use of a Metrabyte Dash-16 A/D interface and custom-designed software.

The effects of clonidine on MAP and HR were analyzed by one-way ANOVA with repeated measures. The effects of clonidine on SNA and RVLM unit activity were analyzed by Kruskal-Wallis ANOVA on ranks. The effects of treatment with anti-DβH-Sap or IgG-Sap on the number of C1 cells and A5 cells and responses to clonidine were analyzed by two-way ANOVA. Student-Newman-Keuls post hoc tests were performed when ANOVA showed a significant effect. Significance was set at $P < 0.05$.

**RESULTS**

**Effects of intravenous clonidine on mean SNA, MAP, and HR.** Intravenously administered clonidine produced the expected dose-related biphasic effect on MAP (Fig. 1, top; 3, 24). At first, clonidine transiently increased AP by activation of peripheral $\alpha_2$-adrenergic receptors. The increases in AP were accompanied by brief dose-related inhibitions of SNA (Fig. 1, middle), attributable to the stimulation of arterial baroreceptors.

After a few minutes, clonidine produced sustained decreases in SNA that reached a steady state within 5–10 min. The highest dose nearly eliminated SNA (Figs. 1, middle, and 2A). These decreases in SNA were accompanied by sustained decreases in MAP (Figs. 1, top, and 2B) and HR (Fig. 2C). Analysis of group data ($n = 7$) for the effects of clonidine at steady state revealed that SNA, MAP, and HR were significantly increased at 1.0 and 1.5 μg/kg, and not to the spinal cord (29, 31). To demonstrate consistency of PNMT immunoreactivity among the groups of rats,
reduced by the two higher doses of clonidine (Fig. 2, A, B, and C).

Which presympathetic RVLM units are sensitive to intravenous clonidine? A presympathetic RVLM unit was recorded in each of the seven animals described above, and an additional 21 presympathetic RVLM units were recorded in 19 rats where SNA was not recorded (in 2 rats, clonidine was given twice separated by 2 h). Although SNA was not recorded in these animals, the responses of the RVLM units and the hypotension produced by clonidine were comparable to those seen in rats with recordings of SNA. With eight of the recorded RVLM units, clonidine was given as a single 10-μg/kg dose. Because this method produced equivalent decreases in AP, HR, and RVLM unit activity compared with the responses seen in rats that received the 10-μg/kg dose in three injections, the data were pooled.

Presympathetic RVLM units have a large range of conduction velocities and heterogeneous phenotypes (15, 21, 28, 31). To determine whether clonidine preferentially affected a subset of these neurons, the data were analyzed with consideration of conduction velocity and catecholaminergic phenotype. The RVLM units were divided into three conduction velocity ranges on the basis of our previous findings (28): less than 1 m/s (unmyelinated C1 cells), 1–3 m/s (lightly myelinated mostly C1 cells), and >3 m/s (mostly non-C1 cells). As shown previously (28), the basal firing rates of the presympathetic neurons were related to their conduction velocities, with the slower conducting neurons having a lower discharge rate (Fig. 2E). Clonidine inhibited most of the recorded presympathetic RVLM neurons (26 of 28). However, two presympathetic RVLM cells showed no change in activity, although the clonidine-induced decreases in SNA were substantial in these cases. On average, presympathetic RVLM neurons of all conduction velocities were comparably inhibited by clonidine (Fig. 2E). However, the degree of inhibition of individual RVLM units was highly variable within each category: 0–83% inhibition in cells with a conduction velocity <1 m/s (n = 13), 0–100% inhibition in cells with a conduction velocity in the 1- to 3-m/s range (n = 10), and 18–78% inhibition in cells with a conduction velocity >3 m/s (n = 5). In addition, the percent inhibition of RVLM units in each category was substantially less than the percent inhibition observed in the SNA (Fig. 2E).

Most of the recorded RVLM units (19/28) were filled with biotinamide to determine whether they contained TH immunoreactivity, a reliable marker for C1 neurons in this region of the RVLM (31). An example of one recorded RVLM neuron that was filled with biotinamide and found to be TH-ir is shown in Fig. 3. Clonidine inhibited both TH-ir and non-TH-ir presympathetic RVLM neurons equally (Figs. 4 and 5). However, the sensitivity of the cells was variable: 14–83% inhibition in TH-ir cells with conduction velocity <1 m/s (n = 7), 23–98% inhibition in TH-ir cells with a conduction velocity >1 m/s (n = 8), and 30–51% inhibition in non-TH-ir cells (n = 4).

Does depletion of bulbospinal catecholaminergic neurons alter responses to clonidine? As expected (29), microinjection of anti-DβH-Sap into the upper thoracic
spinal cord produced massive depletions of the rostral C1 cells (example in Fig. 6). In these treated rats, the number of PNMT-ir cells in the rostral C1 cell group was reduced by 76\% (range 59–95\%) compared with control rats (Table 1). In contrast, the number of PNMT-ir cells in the caudal C1 cell group was unaffected by treatment with anti-DβH-Sap (Table 1), indicating the immunohistochemical detection of C1 cells was comparable between groups. Treatment with anti-DβH-Sap also reduced the number of TH-ir A5 neurons in the ventrolateral pons at every level examined (88 ± 3\%; range 74–95\%). In contrast, treatment with IgG-Sap did not alter the number of A5 neurons (data not shown) or rostral C1 neurons (Table 1).

Responses to clonidine were examined in control rats (n = 6), in rats treated with anti-DβH-Sap (n = 8), and in rats treated with IgG-Sap (n = 6) with the use of the same three-dose protocol (1, 1.5, and 7.5 μg/kg separated by 5 min). In rats treated with anti-DβH-Sap, clonidine produced transient increases in AP and baroreceptor-mediated decreases in SNA and HR (Fig. 7, middle and bottom). These responses were followed by sustained decreases in MAP, HR, and SNA (Figs. 7 and 8) that were not different from control rats or rats treated with IgG-Sap (Fig. 8).

**DISCUSSION**

The present study demonstrates that bulbospinal barosensitive C1 neurons are inhibited by sympatholytic doses of systemically administered clonidine. In addition, barosensitive bulbospinal RVLM neurons with no detectable catecholaminergic phenotype are equally inhibited by clonidine. Massive depletion of bulbospinal C1 cells by anti-DβH-Sap, which would spare the non-C1 presympathetic RVLM neurons, did not alter the sympatholytic or hypotensive effects of clonidine. These data suggest that although both cell types may contribute to the sympatholytic effect of clonidine, the presympathetic C1 cells are not essential for this response. Finally, presympathetic RVLM neurons, regardless of conduction velocity or phenotype, were inhibited to a lesser degree on average than SNA, suggesting that the RVLM is only one of several important central target sites for clonidine.

**Technical considerations.** To determine the necessity of presympathetic C1 neurons in the physiological responses to clonidine, we depleted bulbospinal C1 cells with the use of a newly described immunotoxin, saporin conjugated to an antibody for dopamine β-hydroxylase (16, 29, 37). We have previously shown that microinjection of anti-DβH-Sap into the thoracic spinal cord effectively depletes the rostral C1 cells (29), and coinjection of the retrograde tracer Fast Blue into adjacent spinal levels in these animals indicates that the vast majority of bulbospinal C1 cells is destroyed. Counts of PNMT-ir neurons alone reliably estimate the percent depletion of bulbospinal C1 cells, although on average they underestimate by 12\% due to counts of interspersed PNMT-ir cells that do not project to the cord. Therefore, the average depletion of 76 ± 5\% of rostral PNMT-ir neurons in the present study is likely to be a conservative estimate of the depletion of bulbospinal PNMT-ir neurons.

Anti-DβH-Sap is selective for the depletion of catecholaminergic neurons because the number of bulbospinal non-C1 cells in the RVLM and serotonergic neurons in the adjacent raphe is not affected (29). However, other bulbospinal catecholaminergic neurons including the A5 group are virtually eliminated by systemically administered clonidine. In contrast, treatment with IgG-Sap did not alter the number of A5 neurons (data not shown) or rostral C1 neurons (Table 1).

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conduction velocities. A major difference between the present study and the previous ones is the choice of anesthesia. In the study by Sun and Guyenet (33), rats were anesthetized with halothane, a condition in which the discharge rate of presympathetic RVLM neurons and sympathetic tone are very high. Under these conditions, clonidine is not very efficacious for decreasing SNA (6) or RVLM unit activity (33). The slowly conducting neurons are relatively more inhibited by clonidine under halothane anesthesia, but the responses seen in these neurons are small (18% average inhibition). One speculation of this study was that the apparent preferential inhibition of the slowly conducting cells may be indicative of their catecholaminergic phenotype. Indeed, we have recently shown that the slowly conducting presympathetic RVLM neurons are C1 cells (28), however, the C1 cells are not limited to this range of conduction velocity. Therefore, although the clonidine-responsive cells in the study by Sun and Guyenet (33) were probably C1 cells, many of the unresponsive neurons were also likely to have been C1 cells. In the present study, the clonidine was given in a single 10 μg/kg dose, which produces a decrease in AP and HR comparable to that seen when the same dose is given by 3 injections (as in A). C: this faster conducting (7 m/s) RVLM neuron was not TH-ir, but it was mildly inhibited by clonidine. ↑, Onset of clonidine injections.

![Fig. 4. Examples of clonidine responses of phenotypically identified barosensitive bulbospinal RVLM units.](image)

- **A:** this slowly conducting (0.73 m/s) TH-ir RVLM neuron was transiently silenced during the brief increases in AP produced by clonidine, illustrating the robust barosensitivity of this cell. In contrast, during the sustained decreases in MAP (top) and SNA (middle) produced by clonidine, the RVLM (bottom) unit was only mildly inhibited. The 10 μg/kg dose of clonidine decreased SNA by 80%, but unit activity decreased only 30%. This response was more typical of the units recorded than the example shown in Fig. 1. **B:** this faster conducting (1.8 m/s) TH-ir neuron was also mildly inhibited by clonidine. In this case, the clonidine was given in a single 10 μg/kg dose, which produces a decrease in AP and HR comparable to that seen when the same dose is given by 3 injections (as in A). **C:** this faster conducting (7 m/s) RVLM neuron was not TH-ir, but it was mildly inhibited by clonidine. ↑, Onset of clonidine injections.

![Fig. 5. Summary of sustained responses to 10 μg/kg of clonidine in phenotypically identified barosensitive bulbospinal RVLM neurons.](image)

Cells were classified as TH-ir or non-TH-ir, and TH-ir cells were further classified as slowly conducting (<1 m/s) or faster conducting (>1 m/s). Although responses of units were variable, TH-ir cells of both conduction-velocity ranges and non-TH-ir cells were all equally inhibited by clonidine.

The discharge rate of presympathetic RVLM neurons and sympathetic tone are very high. Under these conditions, clonidine is not very efficacious for decreasing SNA (6) or RVLM unit activity (33). The slowly conducting neurons are relatively more inhibited by clonidine under halothane anesthesia, but the responses seen in these neurons are small (18% average inhibition). One speculation of this study was that the apparent preferential inhibition of the slowly conducting cells may be indicative of their catecholaminergic phenotype. Indeed, we have recently shown that the slowly conducting presympathetic RVLM neurons are C1 cells (28), however, the C1 cells are not limited to this range of conduction velocity. Therefore, although the clonidine-responsive cells in the study by Sun and Guyenet (33) were probably C1 cells, many of the unresponsive neurons were also likely to have been C1 cells. In the study by Allen and Guyenet (3), the rats were anesthetized with urethan, and the RVLM neurons displayed a wider range of inhibitory responses to clonidine (45–100% inhibition). However, the classification of cells that responded with 10–35% inhibition as nonresponding and unidentified phenotypes of the recorded RVLM neurons makes it difficult to assess whether inhibition of presympathetic neurons in the RVLM is restricted to the C1 cells. In the present...
study, we could conclusively demonstrate that both C1 and non-C1 presympathetic neurons are equally likely to be inhibited by clonidine. Therefore, inhibition of presympathetic neurons is not restricted to the C1 cells, and the relative inhibition of C1 and other presympathetic RVLM neurons depends on the state of the animal.

The inhibition of presympathetic RVLM neurons by clonidine presumably has more to do with the nature and strength of their synaptic inputs in a given condition than their phenotype. Indeed, within the RVLM, clonidine exerts its effects by activation of both pre- and postsynaptic receptors (11, 14, 35). Although presympathetic C1 neurons have postsynaptic \( \alpha_2 \)-adrenergic receptors (8), the postsynaptic effects of agonists for these receptors appear to be small and variable, and non-C1 cells have small postsynaptic responses to agonists for \( \alpha_2 \)-adrenergic receptors as well (11, 14). Presynaptic \( \alpha_2 \)-adrenergic receptors appear to play a more important role in the presympathetic RVLM neuron’s response to \( \alpha_2 \)-adrenergic receptor agonists. Analysis by electron microscopy shows that most \( \alpha_2 \)-adrenergic receptors in the RVLM are in axons and axonal terminals that contact both C1 and non-C1 neurons (18). In agreement, patch-clamp recordings in bulbospinal RVLM neurons of rat brain stem slices show that stimulation of \( \alpha_2 \)-adrenergic receptors produces powerful presynaptic inhibition of both glutamatergic and GABAergic inputs (11). Under conditions when most of the synaptic input to RVLM bulbospinal neurons is excitatory, \( \alpha_2 \)-adrenergic agonists would be expected to inhibit these cells strongly. However, the overall effect of clonidine on the activity of presympathetic RVLM neurons will be contingent on the balance of excitatory and inhibitory inputs that these neurons receive under a given condition.

Another factor to be considered in response of the presympathetic RVLM neurons to systemically administered clonidine is the effects on neurons in other brain regions that, in turn, affect the activity of the presympathetic RVLM neurons. Clearly, \( \alpha_2 \)-adrenergic receptors are located in many other areas of the brain that regulate autonomic outflow via the RVLM (34). Clonidine produces a decrease in AP when microinjected into the nucleus of the solitary tract (27). Conversely, clonidine microinjected into the caudal ventrolateral medulla increases AP and renal sympathetic nerve activity, apparently by inhibition of inhibitory inputs to the RVLM (30). These effects antecedent to the RVLM will obviously alter the inputs to the presympathetic neurons and, in turn, modulate the response to clonidine within the RVLM. Therefore, the responses of the presympathetic RVLM neurons to clonidine are the result of a combination of actions on \( \alpha_2 \)-adrenergic receptors within and outside the RVLM.

**Table 1. Effects of treatment with saporin conjugates on the number of C1 neurons in the RVLM**

<table>
<thead>
<tr>
<th>Group</th>
<th>Caudal C1 Cells A-P level</th>
<th>Rostral C1 Cells A-P Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.9 ± 5.1</td>
<td>30.0 ± 7.3</td>
</tr>
<tr>
<td>IgG-Sap</td>
<td>23.9 ± 3.3</td>
<td>35.3 ± 4.4</td>
</tr>
<tr>
<td>Anti-D(\beta)H-Sap</td>
<td>22.2 ± 1.8</td>
<td>8.0 ± 1.2*</td>
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</table>

Values are means ± SE and represent the number of phenylethanolamine-N-methyl transferase immunoreactive (PNMT-ir) neurons/side in the rostral ventrolateral medulla (RVLM) at the corresponding anterior-posterior (A-P) level in millimeters caudal from bregma. PNMT-ir was used as a marker for C1 neurons. Rats treated with saporin conjugated to a mouse IgG (IgG-Sap) showed no differences in the number of PNMT-ir neurons at any A-P level compared with control rats. Rats treated with anti-dopamine \( \beta \)-hydroxylase-saporin (anti-D\(\beta\)H-Sap) showed no difference in the number of caudal PNMT-ir neurons compared with control rats, but they showed a substantial reduction in the number of rostral PNMT-ir neurons compared with control rats and rats treated with IgG-Sap. *P > 0.05 compared with control rats and rats treated with IgG-Sap at that A-P level.
suggests that these neurons contribute to the inhibition of SNA, but it does not provide conclusive evidence. The RVLM contains other types of barosensitive bulbospinal neurons (15, 28), and the relative contributions of C1 and non-C1 cells to the generation of sympathetic vasomotor tone are not known and probably vary with the state of the animal. We have previously demonstrated that depletion of the vast majority of presympathetic C1 neurons by treatment with anti-DβH-Sap does not chronically alter AP in chloralose-anesthetized rats (29). In these rats, the RVLM continues to generate sympathetic vasomotor tone to maintain a normal AP (unpublished observation), suggesting that the non-C1 presympathetic RVLM neurons are fully capable of generating SNA. In the present study, counts of PNMT-ir neurons in the RVLM from two rostral sections showed a 76 ± 5% depletion compared with control rats. This is a conservative estimate of the depletion of bulbospinal C1 neurons, because counts of PNMT-ir neurons would also include some C1 neurons that project to the hypothalamus and not to the spinal cord (29, 31). Nevertheless, because a few C1 cells remained in the RVLM after treatment with anti-DβH-Sap, the possibility that these C1 neurons could account for the persistent clonidine responses in the present study must be entertained. However, on average, the depletion of rostral C1 neurons was quite substantial, and the SNA and AP responses to clonidine in an animal with a 95% depletion of rostral C1 cells were indistinguishable from control rats. Thus the most likely reason for the clonidine-induced inhibition of SNA in rats treated with anti-DβH-Sap is that bulbospinal C1 neurons are not essential for the response. In addition, the depletion of bulbospinal A5 noradrenergic neurons by treatment with anti-DβH-Sap also confirms that these cells are not essential for the sympatholytic and hypotensive effects of clonidine (5). Because the noncatecholaminergic presympathetic RVLM neurons are sensitive to clonidine (Fig. 5) and would not be depleted by intraspinal anti-DβH-Sap, their inhibition likely contributes to the sympathoinhibition and hypotension elicited by clonidine in rats treated with anti-DβH-Sap.

How important is the contribution of RVLM to the sympatholytic effect of clonidine? The findings that effects of systemically administered clonidine can be
mimicked by local microinjection into the RVLM and reversed by microinjection of an \( \alpha_2 \)-adrenergic receptor antagonist into the same site (24) likely overestimate the role of the RVLM. A comparable sympathoinhibition and hypotension may be achieved by the partial inhibition of several sites by intravenous clonidine versus the very effective inhibition of one central site by the local microinjection of clonidine into the RVLM. For instance, in the present study, clonidine produced a relatively modest inhibition of presympathetic RVLM neurons (40% on average) with a dose that nearly eliminated SNA (10 \( \mu g/\text{kg} \)). In contrast, activation of the baroreceptor reflex, which reduces SNA by inhibition of RVLM neurons, produced at least as much inhibition of RVLM unit activity as of SNA (Figs. 1 and 4A; 33). In addition, because microinjection of \( \alpha_2 \)-adrenergic receptor antagonists into the RVLM increases AP, SNA, and RVLM unit activity in the absence of clonidine (12), the apparent reversal of the effects of systemically administered clonidine does not signify that all the effects of systemically administered clonidine occur within the RVLM.

Although the RVLM is not likely to be the sole site of action for the sympatholytic and hypotensive effects of clonidine, it is possible that the role of the RVLM is underestimated by our analysis of changes in RVLM unit activity. Classification of presympathetic RVLM units by conduction velocity or adrenergic phenotype does not reflect the potentially differential targets of these RVLM neurons. The sensitivity of the RVLM neurons to clonidine was highly variable, and although the inhibition of the unit was often less than that observed in the SNA (Fig. 4), some RVLM unit responses closely mirrored the changes observed in the SNA (Fig. 1).

Nevertheless, other studies suggest that clonidine decreases SNA and AP by acting outside the RVLM. Clonidine decreases AP when injected into the gigantocellular depressor area (1). This area also contains spinally projecting neurons that directly innervate sympathetic preganglionic neurons (2). In addition, systemically administered clonidine is likely to inhibit sympathetic preganglionic neurons at the level of the spinal cord. Microiontophoresis of clonidine inhibits the activity of sympathetic preganglionic neurons (13), and microinjection of clonidine into the intermediolateral cell column at T2 of the spinal cord decreases HR (17). In agreement, in slices of spinal cord, clonidine produces postsynaptic inhibition (38) and decreases the evoked release of glutamate onto sympathetic preganglionic neurons (19). Furthermore, in spinally transected animals, SNA evoked by stimulation of somatic nerve (20) or the dorsolateral funiculus of the spinal cord (4) is reduced by systemically administered clonidine. The doses of intravenously administered clonidine that suppress these evoked SNA responses are within the range of those used in the present study. Therefore, it is likely that the sympatholytic and hypotensive effects of clonidine observed in the present study were produced in part by activation of \( \alpha_2 \)-adrenergic receptors in the spinal cord.

In summary, the inhibition of barosensitive bulbar neurons in the RVLM by clonidine has been cited as evidence that these cells are important targets for the sympatholytic effect of this drug (3, 25, 33). The present study demonstrates that in chloralose-anesthetized rats, barosensitive bulbar C1 and non-C1 neurons in the RVLM are inhibited by clonidine in doses that inhibit SNA, suggesting that both cell types may play a role in the decrease in SNA produced by clonidine. Nevertheless, depletion of most presynaptic C1 neurons did not alter effects of clonidine on SNA and AP, suggesting that these responses to clonidine do not require presynaptic C1 cells. Finally, the modest inhibition of presympathetic RVLM neurons by a dose of clonidine that nearly eliminated SNA suggests that other central sites contribute to the inhibitory effects of clonidine. Thus, although central catecholaminergic systems are a target for antihypertensive drugs such as clonidine, the sympatholytic and hypotensive effects of these drugs are likely to be achieved by affecting a variety of cell types via pre- and postsynaptic mechanisms at multiple sites within the central nervous system.

REFERENCES


