Rhythmic Properties of Neurons in the Rostral Ventrolateral Medulla of the Rat In Vitro: Effects of Clonidine

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Granata, Antonio R. and Morton I. Cohen. Rhythmic properties of neurons in the rostral ventrolateral medulla of the rat in vitro: effects of clonidine. J Neurophysiol 88: 2262–2279, 2002; 10.1152/jn.00085.2002. The rostral ventrolateral medulla (RVLM) is thought to be the main central site for generation of tonic sympathetic activity. In the rat in vitro slice preparation, we used intracellular recordings to identify different populations of neurons in the RVLM: 43 spontaneously active neurons with regular (R) or irregular (I) patterns of spike firing and 10 silent neurons. The degree of regularity was quantified by the coefficient of variation (CV = SD/mean) of interspike interval durations, as well as by the rhythmic properties of the spike autospectrum and autocorrelation. The distribution of CVs was clustered: R and I neurons were defined as those with CVs ≤12% (n = 21) or >12% (n = 22), respectively. The R-type and I-type neurons resemble the type II and type I neurons, respectively, which were previously characterized in the RVLM in vivo as barosensitive and bulbospinal. Both types may be important in generation of sympathetic tone. Clonidine (1–100 μM) was applied to 10 R-type neurons and 16 I-type neurons. The firing of 21/26 was depressed to the point of silence. However, 18/26 neurons were excited earlier in the perfusion. The later depression of firing occurred in both I and R neurons and in different cases was associated with either hyperpolarization or depolarization.

INTRODUCTION

The rostral ventrolateral medulla (RVLM) is generally considered as the site of generation of tonic sympathoexcitatory activity (Sun 1995). Barosensitive neurons in the RVLM selectively project to regions in the spinal cord involved in autonomic control (Barman and Gebber 1985). Furthermore, these neurons are considered to form a major contributor of sympathetic-related axons. Although the in vitro slice preparations are at a disadvantage for electrophysiological characterization of medullary sympathetic neurons, they are more suitable than the in vivo preparation for investigating the pharmacological properties of such neurons, because it is possible to accurately control the administration of pharmacological agents during stable intracellular recording. It has long been accepted that adrenergic mechanisms are involved in the central control of cardiovascular function (Kobinger 1978). Moreover, the RVLM has been depicted as an

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important site of action of different pharmacological agents that affect catecholaminergic transmission (Granata et al. 1986) and that are used to treat vascular hypertension, such as the \( \alpha_2 \)-adrenergic agonist clonidine, which is a well-accepted centrally acting antihypertensive agent that binds to \( \alpha_2 \)-adrenergic receptors (Ernserberger et al. 1987). Furthermore, different laboratories have investigated the effect of clonidine on barosensitive bulbospinal neurons in the RVLM, but the results so far have been divergent. For example, in the cat, clonidine applied by iontophoresis inhibited only very few barosensitive bulbospinal neurons in the RVLM (Clement and McCall 1991). On the other hand, in the rat, clonidine inhibited only a subpopulation of bulbospinal barosensitive neurons (Allen and Guyenet 1993). Moreover, the latter research group, using an in vitro slice preparation that included the RVLM, found that clonidine (\( \approx \) 1 \( \mu \)M) did not affect the activity of neurons defined as putative sympathoexcitatory by those authors (Sun and Guyenet 1990).

The pharmacological mechanism responsible for the sympathoinhibitory effect in the RVLM of clonidine and related drugs is still a matter of discussion. Some investigators support the idea that these drugs reduce central sympathetic activity by activating \( \alpha_2 \)-adrenergic receptors (Schmitt et al. 1973). On the other hand, for clonidine as well as other imidazoline derivatives like moxonidine that are effective for treating different forms of the hypertensive syndrome (Prichard et al. 1997; Van Zwieten 1999; Ziegler et al. 1996), it was speculated that they mediate their central sympatholytic effect by activating imidazoline receptors in the RVLM (Ernserberger et al. 1990).

In general, bulbospinal barosensitive neurons in the RVLM are considered to be the sites of action of adrenergic agents mediating central sympathoinhibitory responses (Reis 1996). Yet a critical question to be addressed is: what neuronal type(s) in the RVLM mediate these effects? Adrenergic neurons of the C1 group have direct monosynaptic projections to sympathetic preganglionic neurons in the spinal cord (Milner et al. 1988). Although there is no clear evidence that these neurons are directly involved in generation of tonic sympathetic activity, some investigators have proposed these neurons as the targets of the sympathoinhibitory effect of hypotensive agents like clonidine and other \( \alpha_2 \)-adrenergic agonists (Ruffolo et al. 1993). In addition, it has been proposed that clonidine affects neuronal transmission in the RVLM by activating presynaptic \( \alpha_2 \)-adrenergic receptors (Tingley and Arneric 1990).

The aims of this series of experiments were as follows: first, to further investigate in vitro the electrophysiological characteristics (including the firing properties) of neurons within the RVLM that have similar characteristics to those defined in the same medullary region in vivo; and second, to determine in these characterized neurons the actions of the \( \alpha_2 \)-adrenergic agonist clonidine, as well as the possible block of clonidine effects by the selective nonimidazole \( \alpha_2 \)-adrenergic antagonists rauwolscine and yohimbine. For this purpose, we recorded intracellularly in vitro from neurons located in a coronal medullary slice preparation that included the RVLM.

To quantify the firing patterns of the neurons, in particular with respect to regularity or irregularity, we used time-domain analysis (interspike intervals timing, auto- and cross-correlations) and frequency-domain analysis (autospectra). We formulated quantitative criteria that allowed us to distinguish groups of RVLM neurons having different properties.

**Methods**

**Experimental preparation**

The experiments were done on male Sprague-Dawley rats (90–120 g) anesthetized with ether and decapitated. The brain was rapidly removed from the skull and placed in a dish containing cold Ringer solution (4°C), equilibrated with 95% O\(_2\)-5% CO\(_2\), and containing the following (in mM): 124 NaCl, 5.0 KCl, 1.3 MgSO\(_4\), 1.25 KH\(_2\)PO\(_4\), 2.0 CaCl\(_2\), 26 NaHCO\(_3\), and 10.0 d-glucose. The medulla with the caudal part of thepons was dissected and fixed with cryoacrylic glue in a frontal plane to the cutting chamber of a vibratome (Oxford Instruments). The chamber was then filled with cold (4°C) oxygenated Ringer solution. The medulla was cut into 400–500 \( \mu \)m coronal slices containing the RVLM, which were transferred to an incubation chamber (Medical Systems) and maintained in oxygenated Ringer solution at 25–27°C for approximately 1 h. After that, one slice was transferred to a recording chamber; the bottom surface of the slice was placed on a filter paper held in place on a fine nylon mesh in the chamber, which was continuously superfused with oxygenated Ringer solution at a rate of 0.3–1.0 ml/min at 34 ± 1°C.

**Recordings**

Intracellular recordings were performed with microelectrodes made from borosilicate (2.0 mm OD) filamented glass capillary tubing and filled with a solution of 4–6% biocytin (Sigma) in 0.05 M of KCl or KAcetate, with pH 7.0–7.6. Electrode resistances were 80–170 MΩ. From neurons located between the rostral pole of the RVLM and a plane 500 \( \mu \)m caudally, intracellular potentials were recorded with a low-noise, high-input-resistance bridge amplifier and displayed on a Tektronix (8000 series) oscilloscope for on-line analysis. The signals were also fed into a chart-recorder for monitoring the membrane potential and membrane input resistance, and into a digital oscilloscope (Nicolet), at sampling intervals of 2–50 \( \mu \)s with 12-bit precision, at a maximum of 16K data points per record. Continuous recordings were taken into a video cassette recorder via a digital interface (NeuroData) at a sampling rate of 22 or 44 kHz.

**Current application**

During the recording of the continuous data stream, we made intermittent applications of trains (repetition rate, 0.5–1.0 Hz) of depolarizing or hyperpolarizing current pulses (380-ms pulse duration) with graded magnitudes. The neuronal membrane input resistance was calculated from the magnitudes of the current pulses and of the evoked intracellular voltage changes, which determined the current/voltage relationship (as displayed in Fig. 3F).

**Off-line analysis**

Signals were digitized by an A/D converter (RC Electronics) at a sampling rate of 2,500 or 5,000 Hz (0.4-ms or 0.2-ms bin duration, respectively) and transferred into the hard disk of a PC. Pulses (duration 0.4 ms or 0.2 ms) were derived from the spikes by a time-amplitude discriminator (WPI). By means of a customized program, portions of the digitized data stream were viewed on the monitor, and sections that did not include current pulses were marked to be used for data analysis. In long recordings obtained during drug perfusion, segments occurring at various times after start of perfusion were marked for later analysis of the time course of drug effects. The digitized data in these time slices (duration range, 30–100 s) were subjected to time-domain and frequency-domain analysis.

**Interspike interval analysis**

The interspike intervals in each data segment were measured, and the values of successive intervals were plotted (Fig. 1) to verify the
stationarity of the sample. If the display indicated nonstationarity, as shown by the existence of a trend, another sample was chosen from the original data. The regularity of discharge of the spikes in the sample was ascertained by computation of the coefficient of variation (CV = SD/mean) of the interval distribution. Obviously, a nonstationary sample would furnish an incorrect value of the CV, because of the continuous change with time of the interval durations.

Correlation analysis

Autocorrelation histograms (ACHs) of spike-derived pulses and cross-correlation histograms (CCHs) of pulses versus membrane potential (MP) were computed. The correlograms were normalized using the square root of the variance of each signal (Bendat and Piersol 1986). The spike-MP CCH furnished information on the temporal relation between spike occurrence and the slower depolarizing and hyperpolarizing change of MP; for this purpose, the inclusion of both positive and negative lags in the display was suitable.

Spectral analysis

The autospectrum of a neuron’s spike activity was computed from a data array derived by subjecting the spike-derived pulses to a low-pass filtering operation (effective sampling rate 500 Hz) by means of the sinc function (Christakos et al. 1991). The final spectra were obtained by averaging the spectra computed from data windows of 2.048-s duration, using a standard fast Fourier transform algorithm (Bendat and Piersol 1986). The number of windows (range, 5–100) was determined by the duration of the data sample. With these parameters, the spectral frequency resolution was 0.49 Hz/bin. The autospectral strength was initially quantified as relative power, defined as the power in each frequency bin divided by total power (Bendat and Piersol 1986). Several variables obtained from the autospectra were used to characterize rhythmicity of firing: peak autospectral (fundamental) frequency, number of harmonic peaks, and the rhythmicity index.

Rhythmicity index

For each data sample, this index was calculated as follows (Fig. 1): The baseline power was calculated as the mean of the power in the flat portion of the autospectrum (i.e., the portion after decay of rhythmicity). The peak power was defined as the amplitude of the peak autospectral (fundamental) frequency, and the rhythmicity index was defined as (peak power)/(baseline power). This index is similar to the

![Image of the methods for assessing variability of firing by a neuron in the RVLM in vitro. Recordings were taken in 2 different states: 1) during undisturbed spontaneous activity (A1 and B1) and 2) during a period of clonidine (1 μM) perfusion starting 858 s after perfusion onset (A2 and B2). Top panels: successive interspike interval durations (●) shown for each condition. Thick line, mean interval duration; thin lines, mean ± SD. A1: 55.5 ± 16.1 ms. A2: 66.0 ± 3.0 ms. Coefficient of variation (CV = SD/mean): A1: 29.0%; A2: 4.5%. Thus the neuron’s firing pattern changed from irregular (I) in state 1 to regular (R) in state 2. Note that the ordinate scales differ between A1 and A2. Bottom panels: method for calculating the rhythmicity index, a measure of degree of regularity of firing. Autospectra were computed from spike-derived pulses using windows of 2.048-s duration, with sampling rate of 500 Hz and consequent spectral resolution of 0.49 Hz/bin. In each spectrogram, the horizontal lines near the bottom represent the mean ± SD of the power in the bins comprising the BASELINE (flat portion of the spectrogram after decay of rhythmicity), which is marked by short vertical lines. The power ratio at each frequency = (bin power)/(baseline power). The rhythmicity index, defined as the power ratio at the peak (fundamental) frequency, is indicated by a short horizontal line. Number of windows: B1: 16; B2: 50. Number of spikes: B1: 596; B2: 1,545. Mean firing rate (spikes/s): B1: 18.2; B2: 15.1. Rhythmicity index: B1: 3.6 at 19.0 Hz; B2: 10.1 at 15.1 Hz.
Identification of the recorded neurons

Some of the recorded neurons were intracellularly labeled with biocytin (n = 52) by passing 4- to 6-nA negative rectangular pulses of 150-ms duration at 3.3 Hz for 6–10 min. After recording, the slice containing the injected neuron(s) was transferred to a fixative solution of 4% paraformaldehyde and 1.5% picric acid in phosphate buffer saline (PBS) at pH 7.4, and stored at 4°C overnight. After that, the slice was transferred to a solution of 30% sucrose in 0.15 M PBS for 4 h and then sectioned on a freezing microtome into 50-μm-thick sections, which were then rinsed several times in 0.1 M phosphate buffer (pH 7.4) and processed for histofluorescence in the intracellularly labeled neuron(s). Histological sections were incubated for 4 h in avidin-Texas Red 1:200 in phosphate buffer solution plus 0.1% triton X100 and 1% sodium azide. At the end of the incubation, the tissue was rinsed several times in a fresh solution of phosphate buffer for 1 h and the sections were mounted in a solution of glycerol in phosphate buffer (1:3) plus 1% n-propyl gallate. Sections were examined under an epifluorescence microscope with a filter combination for rhodamine (G-2A). The location of a filled neuron was determined and marked on a page from a histological atlas (Paxinos and Watson 1986).

Drug administration

Drugs used in this study [clonidine hydrochloride (1–100 μM), rauwolscine hydrochloride (10 μM), and yohimbine hydrochloride (10 μM)] were dissolved in Ringer solution, which was applied by a gravity perfusion system.

RESULTS

Characterization of different neuronal types

In in vitro slice preparations of the rat (n = 17), intracellular recordings were obtained from 43 spontaneously firing neurons and 10 silent neurons. These were found in a region of the RVLM extending from the caudal border of the facial nerve nucleus to a plane 500 μm caudal, and extending from the medial border of the spinal nucleus of the trigeminal nerve to the lateral border of the nucleus paragigantocellularis (Fig. 2). The average number of neurons recorded per preparation was about three (range, 1–6). The recorded neurons had resting MP between −43 and −85 mV. The membrane input resistance was measured by injections of intracellular current and ranged from 32 to 218 MΩ.

The spontaneously active neurons could be further classified into two groups: 1) neurons with a very regular (R) pattern of action potential discharge and 2) neurons discharging action potentials in an irregular (I) fashion, which had substantial spontaneous postsynaptic potentials. The criterion for designating a neuron as R-type or I-type was the CV of interspike intervals as described in detail below. The mean MP for the R group was −54.74 ± 1.82 (SE) mV, and for the I group was −52.31 ± 1.25 mV; this difference was not statistically significant.

The analysis of properties of a typical regularly firing R-type neuron is shown in Fig. 3. Neurons of this type had a spike afterhyperpolarization consisting of an initial rapid component, followed by a slower hyperpolarized component, which in turn was followed by a slow depolarizing potential that drove the membrane potential to the threshold for action potential generation (Fig. 3, A and B). This pattern can be seen in the spike-MP CCH of Fig. 3D.

When the firing of R-type neurons was suppressed by a hyperpolarizing pulse, after cessation of the pulse the rhythm resumed with regular interspike intervals such as those occurring before current injection (Fig. 3A); this effect is similar to that observed in vivo for barosensitive type II neurons (Granata and Kitai 1992). Furthermore, EPSPs were not observed during hyperpolarization, whether it was produced by negative current pulses (Fig. 3A) or by continuous negative current sufficient to bring the membrane below the spike threshold level.

Another feature commonly found in R-type neurons (14/18 tested with hyperpolarization pulses) was an initial relatively fast-developing anomalous rectification, indicated by a decay in the hyperpolarization (Fig. 3A). In this sample of R-type neurons, the current/voltage relationship measured at the initial peak of the hyperpolarization (before activation of the timedependent inward rectification) was found to be linear up to a range of −30 to −50 mV (as in Fig. 3F).

The regularity of spike firing is indicated in the spike ACH (Fig. 3C) and autospectrum (Fig. 3E) by the occurrence of numerous narrow peaks (fundamental and harmonics) and was quantified by the CV of interspike intervals, which was 3% for this neuron.

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Similar analyses for a typical irregularly firing (I-type) neuron are shown in Fig. 4. The pattern of spike afterhyperpolarization is shown in the spike-MP CCH (Fig. 4D). During application of hyperpolarizing current pulses, the neuron’s MP had spontaneous postsynaptic potentials (Fig. 4, A and B) that increased in magnitude when the neuron was hyperpolarized (Fig. 4B); at the offset of the hyperpolarizing pulses, irregular firing resumed. During constant hyperpolarization (applied to allow increased magnitude of spontaneous EPSPs), depolarizing current pulses elicited spike firing (Fig. 4B). I-type neurons only rarely (4/19 tested) exhibited anomalous rectification when the membrane potential was hyperpolarized by negative current pulses of magnitude comparable to those applied to R-type neurons (Fig. 4, A and B). In 13 of 18 I-type neurons tested, the current/voltage relationship showed an inward rectification when the membrane was hyperpolarized more than \(-15\) to \(-20\) mV from resting membrane potential (Fig. 4F).

The limited rhythmicity of spike firing for this I-type neuron is indicated in the unit ACH (Fig. 4C) and the autospectrum (Fig. 4E) by the occurrence of only a single autospectral peak (i.e., absence of harmonics) and by the relatively high value (31%) of the CV of interval durations.

The CV of interspike intervals was adopted as a criterion of degree of regularity of a neuron’s firing. To verify the stationarity of the interval distribution during the spike train, a plot was made of successive values of interval duration, as in Fig. 1, where the flatness (lack of trend) indicates stationarity.

For the sample of 43 spontaneously firing neurons, the CVs ranged from 3% to 40%. The plots of Fig. 5 show the relation between several discharge variables and the CV for the sampled population. As seen in Fig. 5A, the rhythmicity index, calculated from the unit autospectrum as in Fig. 1, for the 43 neurons ranged from 21.0 to 1.5, a higher index being associated with smaller CV. The plot seems to indicate separation between regions of lower and higher CV near CVs of 10–12%.

To obtain a more exact indicator of such separation, we plotted the cumulative distribution (range, 0.0–1.0) of the rhythmicity index (CUM. RHY. INDEX) versus CV values, as shown in Fig. 5B. There seems to be an inflection point (change of slope) at about CV = 12%. Therefore two linear regression lines were computed for values of CV \(\leq 12\%\) versus \(>12\%\), respectively. The slopes, calculated as CUM. RHY. INDEX/CV, were as follows: left = 0.0679 and right = 0.0078. It can be seen that there is a marked difference of slope between the two regression lines (left/right slope = 8.70). Therefore the criterion for separation of two regions was set at 12% CV (indicated by vertical lines), with neurons having CV \(\leq 12\%\) being designated as regular (R; \(n = 21\)), and neurons having CV >12% being designated as irregular (I; \(n = 22\)).

An inverse relation also existed between CV and number of autospectral peaks (fundamental and harmonics; Fig. 5C), as well as between CV and the frequency value of the main (fundamental) autospectral peak (range, 4–34 Hz; Fig. 5D). However, the latter distribution was more scattered than the other distributions (Fig. 5, A and C). Nevertheless, the difference of spectral frequencies between the R and I groups

FIG. 3. Analysis of intracellular recording from a regularly firing neuron in the RVLM in vitro. A: effect of hyperpolarization of the neuron by a negative current pulse applied through the recording electrode. The hyperpolarization interrupted spontaneous activity, and after the end of the current pulse, the pacemaker-like activity resumed. Note the absence of postsynaptic potentials (PSPs) during hyperpolarization. The 2-phase voltage deflection during the hyperpolarization period is due to anomalous rectification. B: isolated spike (truncated in display), which had an action potential duration of 1.6 ms. The spike is preceded by a slow depolarizing potential and followed by an afterhyperpolarizing potential. C: autocorrelation histogram (ACH) of spike-derived pulses, from a sample of 207 spikes during 11.9 s; note strong periodicity. D: cross-correlation histogram (CCH) of spike-derived pulses vs. membrane potential (MP); spike deflection truncated in display. E: autospectrum of spike-derived pulses from the same sample, computed from 178 spikes in 5 windows of 2048-s duration. Horizontal line indicates baseline power ratio (=1.0). Rhythmicity index: 15.7 at 17.3 Hz. Note the similarity of the autospectrum and ACH. F: current/voltage relationship for the neuron.
(15.60 ± 1.58 vs. 10.42 ± 1.18 Hz, respectively) was statistically significant (P < 0.02).

The mean firing rate (number of spikes/sample time) was usually close (within 0.5 Hz) to the frequency value of the fundamental autospectral peak. This was due to the presence of a modal interval corresponding to the peak autospectral frequency.

The silent neurons (n = 10) were induced to fire action potentials (with irregularly distributed intervals) by injecting depolarizing current (Fig. 6B). These neurons had resting membrane potentials that ranged from −50 mV to −72 mV, with a mean value of −61.5 mV. This value was more negative than that observed in either R-type or I-type neurons, as mentioned earlier in this section, and it was statistically significant for both types (t-test, P < 0.001). The silent neurons had longer action potential durations (Fig. 6A) than the spontaneously firing neurons (Fig. 3B): 4.20 ± 0.30 ms vs. 1.68 ± 0.50 ms, respectively. This difference was statistically significant (t-test, P < 0.001). There was no significant difference of action potential duration between the R-type and I-type neurons. The current/voltage relationship in silent neurons (Fig. 6C) was linear ≤20 mV below the spontaneous membrane potential in nearly all neurons tested.

The silent neurons were localized in an area close to the ventral medullary surface (<100 μm), whereas spontaneously firing neurons were broadly distributed in the RVLM. The morphology of five biocytin-labeled silent neurons showed somata predominantly polygonal in shape. On the other hand, the labeled cell bodies of spontaneously active neurons of either R-type (n = 6) or I-type (n = 7) were fusiform, oval, and polygonal in shape. The size of the cell bodies was similar for both silent and spontaneously active groups, with ranges of 15–55 μm in the long axes and 10–28 μm in the short axes.

**Effects of clonidine on neurons of different types**

After an 8- to 12-min period of recording from spontaneously firing neurons (to verify stability of recording conditions), clonidine (1–100 μM) was applied by superfusion while continuously recording from 26 RVLM neurons (10 of R-type and 16 of I-type). The onset of change of membrane potential produced by clonidine varied between different neurons (range, 55–220 s from the start of perfusion). For 21/26 neurons, there was eventually almost complete suppression of firing after a time of perfusion of 180–2100 s. For 18 neurons, there was an initial excitation (increase of discharge frequency and increased rhythmicity of discharge). Several patterns of response were observed, as summarized in Table 1. Several different patterns of response to clonidine could be found in each individual slice.

**Regularly firing neurons**

For R-type neurons (Table 1, left pair of columns), the most common pattern of response to clonidine (1 μM or 10 μM), found in six neurons (example in Figs. 7–9), was first a short-lasting moderate hyperpolarization (3–5 mV from the control membrane potential) and increase in input resistance.
After that, the neuron started to depolarize, the discharge frequency increased, and the membrane input resistance decreased (Figs. 7B and 8B). Subsequently, the neuron became further depolarized (10–18 mV from the control membrane potential), and now the discharge frequency was reduced (Fig. 7C and D). Finally, the neuron became more depolarized (8–12 mV from the previous level shown) and almost ceased firing (Figs. 7E and 8C). At this point, the anomalous rectification was abolished, and the membrane input resistance was moderately increased (Fig. 8D, ○) over the control level (Fig. 8D, ●). After washout with normal Ringer solution, the changes were partially reversed (Fig. 7F).

The effects of clonidine on the neuron’s rhythmicity over the time course of perfusion are shown in the unit autospectra and spike-MP CCHs of Fig. 9. The fundamental autospectral frequency in the control state was 14.2 Hz (Fig. 9A), with prominent rhythmicity, as indicated by the presence of many harmonic peaks and a value of 7% for the CV of interval duration. The excitation in the earlier portion of clonidine perfusion

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

**FIG. 5.** Relations between rhythmic firing properties and the CV of interval durations for the population of studied neurons (n = 43). In each graph, for each neuron, the value of a discharge variable is plotted against the CV of interval durations in the recorded sample. A: rhythmicity index (RHY. INDEX, defined in METHODS). B: cumulative distribution (range: 0.0–1.0) of values of rhythmicity index (CUM. RHY. INDEX). Vertical line at CV = 12.2% indicates separation between regions that specify neurons as regular (R; left, n = 21) or irregular (I; right, n = 22). The slopes calculated as CUM. RHY. INDEX/CV were as follows: R (left) = 0.0679 and I (right) = 0.0078. Note marked difference between slopes of regression lines (left/right slope = 8.70) for the 2 subsamples. C: number of distinct autospectral peaks (fundamental plus harmonics). D: peak autospectral (fundamental) frequency.

After that, the neuron started to depolarize, the discharge frequency increased, and the membrane input resistance decreased (Figs. 7B and 8B). Subsequently, the neuron became further depolarized (10–18 mV from the control membrane potential), and now the discharge frequency was reduced (Fig. 7C, D). Finally, the neuron became more depolarized (8–12 mV from the previous level shown) and almost ceased firing (Figs. 7E and 8C). At this point, the anomalous rectification was abolished, and the membrane input resistance was

![Graph A](image5)

![Graph B](image6)

![Graph C](image7)

**FIG. 6.** Analysis of intracellular recording from a silent neuron. A: action potential (duration, 4.6 ms) evoked by injection of depolarizing current; note succeeding afterhyperpolarizing potential. B: action potentials (truncated in display) during depolarizing current pulses of different magnitudes. C: current/voltage relationship for the neuron.
TABLE 1. Responses to clonidine shown by regularly and irregularly firing neurons in RVLM in vitro

<table>
<thead>
<tr>
<th>Type of response</th>
<th>Type-R Neurons</th>
<th>Type-I Neurons</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Clon (1 (\mu)M)</td>
<td>Clon (10 (\mu)M)</td>
</tr>
<tr>
<td>A Type of response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early depolarization/excitation, followed by hyperpolarization/inhibition</td>
<td>9, n = 0</td>
<td>4, n = 0</td>
</tr>
<tr>
<td>Early depolarization/excitation, followed by further depolarization and ending in partial to total depression of firing</td>
<td>2, n = 0</td>
<td>4, n = 0</td>
</tr>
<tr>
<td>Inhibition by maintained hyperpolarization</td>
<td>2, n = 0</td>
<td>0, n = 0</td>
</tr>
<tr>
<td>Depolarization/excitation</td>
<td>1, n = 0</td>
<td>0, n = 0</td>
</tr>
<tr>
<td>No effect</td>
<td>1, n = 0</td>
<td>0, n = 0</td>
</tr>
<tr>
<td>Clon (1 (\mu)M) + Rauw (10 (\mu)M)</td>
<td>3, n = 3</td>
<td>0, n = 0</td>
</tr>
<tr>
<td>Clon (1 (\mu)M) + Yohim (10 (\mu)M)</td>
<td>0, n = 0</td>
<td>1, n = 1</td>
</tr>
</tbody>
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In A, clonidine (Clon) was perfused alone; in B, in the presence of rauwolscine (Rauw); in C, in the presence of yohimbine (Yohim). \(n\), number of neurons. RVLM, rat rostral ventrolateral medulla.

encompassed (I) increase of autospectral frequency to 21.0 Hz after 410 s (Fig. 9B) and to 23.4 Hz after 683 s (Fig. 9C) and 2) increase of rhythmicity index from 6.4 in Fig. 9A to 9.4 in Fig. 9B and to 10.4 in Fig. 9C. Thereafter, during the continued depolarization, there was depression of activity, indicated in Fig. 9D (1,206 s after start of perfusion) by reduction of spectral frequency to 5.4 Hz and reduction of rhythmicity index to 2.9. After the sample of Fig. 9D, with continued clonidine infusion the neuron became silent (Figs. 7C and 8C). Finally, after washout with normal Ringer solution, the changes were partially reversed, as indicated in Fig. 9E by resumption of discharge, with a peak autospectral frequency of 6.3 Hz and rhythmicity index of 4.5. The spike-MP CCHs (Fig. 9, right panels) show a MP pattern similar to that in Fig. 3: postspike afterhyperpolarization followed by a slow depolarizing potential.

A somewhat different pattern of response to clonidine was shown by two other R-type neurons: there was no excitation, but rather only hyperpolarization was elicited (750–960 s after clonidine infusion). Finally, for another R-type neuron, clonidine elicited only depolarization and excitation; and for still another R-type neuron, clonidine had no effect. It might be relevant that this last neuron had a spontaneous autospectral peak (at 34 Hz), which was the highest in the sampled population.

Irregularly firing neurons

Several patterns of response were observed. In six I-type neurons (Table 1, right columns; example in Figs. 10–12),...
clonidine (1 μM or 10 μM) first produced a transient (520–650 s after start of clonidine infusion) membrane depolarization (3–5 mV from the control membrane potential), with increased discharge frequency and a 10–20% increase in membrane input resistance (Figs. 10B and 11B). After that (1400–1650 s after start of clonidine infusion), the membrane potential changed in the opposite direction, becoming hyperpolarized (4–6 mV from the control membrane potential) with substantial reduction of discharge frequency and a prolonged augmentation in input resistance (Fig. 10C). Finally, we observed a further hyperpolarization and reduction in firing frequency while the membrane resistance remained elevated (Fig. 10, D and E, and Fig. 11, C and D).

The effect of clonidine on the neuron’s rhythmicity over the time course of perfusion are shown in the unit autospectra and spike-MP CCHs of Fig. 12. The neuron had relatively weak rhythmicity in the control state (Fig. 12A), as indicated by the presence of only one autospectral peak at 16.6 Hz. Clonidine initially (Fig. 12B, 540 s after start of perfusion) produced only a moderate excitation (spectral frequency increase to 22.0 from 16.6 Hz) with little change of rhythmicity index (from 3.4 to 3.8). Thereafter (Fig. 12C, 1500 s after start of infusion) there was depression of activity due to hyperpolarization, with decrease of peak spectral frequency (from 22.0 to 12.3 Hz) and rhythmicity index (from 3.8 to 2.2). After further infusion (Fig. 12D, 1700 s after start), with additional hyperpolarization there was complete loss of rhythmicity, as indicated by the flatness of the autospectrum, together with further decrease of mean firing rate (to 2.2/s from 12.1/s in Fig. 12C). Finally, after 450 s of drug washout (Fig. 12E), there was partial recovery: increase of mean firing rate (from 2.2/s to 3.9/s) but no recovery of rhythmicity. The spike-MP CCHs (Fig. 12, right panels) show a MP pattern similar to that in Fig. 4: spike afterhyperpolarization followed by recovery of membrane potential.

In another type of response, shown by three I-type neurons, clonidine (1 μM or 10 μM) also initially elicited depolarization with increase in firing rate and spectral frequency, but thereafter, instead of hyperpolarization, there was further depolarization accompanied by a dramatic decrease of firing frequency and decrease of membrane input resistance. This kind of response was evoked in two additional I-type neurons by infusion of 100 μM clonidine. In another four I-type neurons, clonidine (1 μM or 10 μM) did not elicit an early excitation but did elicit the hyperpolarizing response with a marked decrease of firing rate and increase of input resistance. A similar type of response was evoked in one additional I-type neuron by infusion of 100 μM clonidine. In two I-type neurons, clonidine (1 μM or 10 μM) evoked only a moderate depolarization and increase in firing rate. Finally, in one I-type neuron, clonidine did not produce any effect.

For both R-type and I-type neurons, clonidine did not change the duration or shape of the action potentials.

For 21/26 neurons, clonidine perfusion (1–100 μM) eventually produced depression of firing to the point of silence. In addition, 18/26 neurons were excited earlier in the perfusion.

**Fig. 8.** Effects of clonidine (1 μM) on the R-type neuron of Fig. 7, shown on a fast timebase. A: control (before drug application). MP at the half point of the slow depolarizing potential was −52 mV. B: recording is during the time slice of Fig. 7B, when there was depolarization (−47 mV) and increased firing rate. C: recording is during the time slice of Fig. 7C, when there was further depolarization (to −47 mV), increase of membrane resistance, and cessation of firing. D: current/voltage relationship showing the increase of membrane resistance in the presence of clonidine (○) compared with control values (●).
FIG. 9. Frequency-domain and time-domain analysis of the time course of clonidine effects on firing rhythmicity of the R-type neuron of Fig. 7 during times when no current was applied. Spike autospectra (left column) and spike-MP CCHs (right column): same formats as in Fig. 3. A: control. Note strong rhythmicity of discharge (autospectral peak at 14.2 Hz followed by 3 harmonic peaks). B–D: “time” denotes time from onset of clonidine perfusion to start of record used for computations. B: time, 410 s. Note increase of rhythmicity as shown by increased value of peak frequency (from 14.2 to 21.0 Hz), number of harmonics, and rhythmicity index (from 6.4 to 9.4). C: time, 683 s. Note little change of rhythmicity. D: time, 1,206 s. Note marked reduction of peak frequency (from 23.2 to 5.4 Hz) and of rhythmicity index (from 10.4 to 2.9). E: after drug washout; record starts 1,800 s after start of washout. Note partial recovery of rhythmicity, as shown by increased peak frequency value (from 5.4 to 6.3 Hz) and rhythmicity index (from 2.9 to 4.5). Number of windows (each of 2.048-s duration): A: 30; B: 14; C: 27; D: 31; E: 43. Number of spikes: A: 858; B: 599; C: 1,285; D: 544. Mean firing rate (spikes/s): A: 14.0; B: 20.9; C: 23.2; D: 5.1; E: 6.2. Rhythmicity index: A: 6.4 at 14.2 Hz; B: 9.4 at 21.0 Hz; C: 10.4 at 23.4 Hz; D: 2.9 at 5.4 Hz; E: 4.5 at 6.3 Hz.
The later depression occurred in both R-type and I-type neurons and was associated with hyperpolarization/inhibition in 12 neurons and with depolarization in 9 neurons. Thus the predominant effect of clonidine was eventually depression of firing to the point of silence.

Effects of α-adrenoceptor antagonists on clonidine responses

We studied the actions of the α2-adrenoceptor antagonists rauwolscine (4 neurons) and yohimbine (2 neurons) on the effects produced by clonidine. In all cases, the blockers were perfused alone for a period of 15–20 min before perfusing the slice with a solution containing both the agonist and the antagonist.

For three R-type neurons (example in Figs. 13 and 14), rauwolscine (10 μM) applied in the superfusate elicited a moderate depolarization and increase of firing rate without change of membrane input resistance. This moderate excitation is indicated by the changes in spectral variables seen in Fig. 14A (control) versus Fig. 14B (910 s after start of rauwolscine perfusion): increase of peak autospectral frequency from 6.8 to...
FIG. 12. Frequency-domain and time-domain analysis of the time course of clonidine effects on firing rhythmicity of the I-type neuron of Fig. 10 during times when no current was applied. Spike autospectra (left column) and spike-MP CCHs (right column): same formats as in Fig. 3. A: control. Note moderate rhythmicity of discharge (autospectral peak at 16.6 Hz followed by 1 harmonic of moderate amplitude).

B: time, 540 s. Note increase of peak frequency (from 16.6 to 22.0 Hz) and moderate change of rhythmicity index (from 3.4 to 3.8).

C: time, 1,500 s. Note marked decrease of peak frequency (from 16.6 to 12.3 Hz) and rhythmicity index (from 3.8 to 2.2).

D: time, 1,700 s. Note disappearance of rhythm, but the neuron continues to fire at a low rate (2.2/s).

E: after drug washout; record starts 450 s after start of washout. There was partial recovery of mean rate (to 3.9/s) but no recovery of rhythm. Number of windows (each of 2.048-s duration): A: 19; B: 34; C: 14; D: 15; E: 18. Number of spikes: A: 627; B: 1,519; C: 353; D: 67; E: 143. Mean firing rate (spikes/s): A: 16.1; B: 21.8; C: 12.1; D: 2.2; E: 3.9. Rhythmicity index: A: 3.4 at 16.6 Hz; B: 3.8 at 22.0 Hz; C: 2.2 at 12.3 Hz; D and E: not applicable (no autospectral peaks).
9.8 Hz and moderate increase of rhythmicity index from 5.6 to 6.5. Subsequent application of clonidine (1 $\mu$M) in the presence of rauwolscine did not produce the usual inhibitory effect on firing (Fig. 13, A and B), i.e., rauwolscine blocked the inhibition. However, in the presence of rauwolscine, there was still a moderate excitation by clonidine, as indicated in Fig. 14, B–D: increase of rhythmicity index from 6.5 in Fig. 14B to 7.9 in Fig. 14C (286 s after clonidine) to 8.8 in Fig. 14D (after an additional 428 s of clonidine perfusion). Finally, after 1,200 s of washout of both agents (Fig. 14E), there was a tendency toward reversal of the moderate excitatory effects of both agents, as can be seen by comparison of Fig. 14E (after washout) with Fig. 14A (before drug application). The spike-MP CCHs (not displayed) for this neuron show that there was little difference of MP pattern (except for a moderate change of spectral frequency) between the condition after rauwolscine infusion (Fig. 14A) and after subsequent clonidine infusion (Fig. 14B).

Finally, for one I-type neuron, the inhibitory effect of clonidine (1 $\mu$M) was not blocked by prior infusion of rauwolscine (10 $\mu$M).

The effects of the antagonist yohimbine were analyzed for two I-type neurons. The example of Fig. 15 shows that yohimbine (10 $\mu$M) did not block the inhibitory effects of clonidine (1 $\mu$M), since the increased negativity of membrane potential, increase of membrane resistance, and decrease of firing rate produced by clonidine still occurred after prior application of yohimbine. Similar results were obtained in the second neuron.

**Fig. 13.** Effects of clonidine (1 $\mu$M) plus rauwolscine (10 $\mu$M) perfusion on an R-type neuron. The format of traces is similar to that in Fig. 7. A: recording after 190 s of perfusion with rauwolscine. B: recording starts 960 s after clonidine perfusion onset together with continuing rauwolscine perfusion. MP, firing properties, and membrane input resistance were practically unchanged. Thus the depressant effect of clonidine was completely blocked by rauwolscine.

**Fig. 14.** Frequency-domain analysis of the time course of rauwolscine and clonidine effects on firing rhythmicity of the R-type neuron of Fig. 13 during times when no current was applied. Spike autospectra with same format as in Fig. 3. A: control. Note strong rhythmicity of discharge with autospectral peak at 6.8 Hz followed by 5 harmonics. B: after 910 s of rauwolscine perfusion, the peak autospectral frequency increased to 9.8 Hz. C: after 286 s of clonidine perfusion with continued rauwolscine perfusion, there was only a moderate change of frequency and amplitude of the rhythm. D: after another 428 s of rauwolscine and clonidine perfusion, there was again little change of pattern. E: after 1,135 s of washout of both agents, there was a decrease in the strength of the rhythm as shown by the decreased peak spectral frequency (from 11.2 to 6.3 Hz) and a decreased number of harmonics. Number of windows (each of 2.048-s duration): A: 15; B: 37; C: 37; D: 31; E: 14. Number of spikes: A: 213; B: 727; C: 785; D: 721; E: 185. Mean firing rate (spikes/s): A: 6.9; B: 9.6; C: 10.4; D: 11.4; E: 6.5. Rhythmicity index: A: 5.6 at 6.8 Hz; B: 6.5 at 9.8 Hz; C: 7.9 at 10.3 Hz; D: 8.8 at 11.2 Hz; E: 3.9 at 6.3 Hz.
DISCUSSION

Types of neuronal discharge

The results presented in this study support our earlier proposal (Granata 1995; Granata and Kitai 1992) that the RVLM contains two different types of spontaneously active neurons. A first group exhibits a very regular pattern of action potential discharge, whereas a second group has an irregular pattern of firing and shows spontaneous postsynaptic potentials. A third group of neurons is normally silent, and action potentials occur only during neuronal depolarization elicited by intracellular injection of positive current.

In the study of sympathetic neural discharge, the existence of rhythms in population and unit discharges, at both peripheral and central levels, has merited the attention of researchers (Barman and Gebber 2000). These rhythms are pertinent as factors that could aid the study of mechanisms producing sympathetic tone. In studies of the rat in vivo, the presence of rhythms locked to the cardiac cycle (presumably via baroreceptor afferents) in brain stem unit discharges has served to identify those units as being involved in sympathetic activity (Granata and Kitai 1992; Sun et al. 1988a; Zagon and Spyer 1996), but there have been no reports of other types of fast rhythm, such as the “10-Hz” rhythm found in the cat (Barman and Gebber 2000).

Another type of rhythm in RVLM neurons of the rat is a regular discharge that was found after kynureenate application (Sun et al. 1988a) and was designated as “pacemaker-like”. In addition, similar unitary rhythms have been found in in vitro preparations, such as brain-stem/spinal cord preparations (Oshima et al. 2000) and the medullary slice preparation (Granata 1995; Kangrga and Loewy 1995; Lewis and Coote 1993; Piguët and Schlichter 1998; Sun and Reis 1994; Sun et al. 1988b).

In the original report on regularly firing neurons (Sun et al. 1988a), a neuron’s discharge was characterized as regular if there was marked periodicity in its spike-triggered interval histograms (a variant of autocorrelation). The designation of regularity was also based on examination of superimposed spike-triggered oscilloscope sweeps (Granata and Kitai 1992) as well as on plots of the n’th versus the (n + 1)’th interval duration (Lewis and Coote 1993). However, in other reports (Oshima et al. 2000; Piguët and Schlichter 1998), no quantitative criterion for regularity was stated. Moreover, none of the published reports quantify the degree of firing irregularity. Therefore, in this study, we developed methods for quantifying the degree of regularity or irregularity of a neuron’s discharge.

We used as the primary criterion for regularity the CV of the neuron’s interspike interval duration during a stationary period of discharge. (The requirement for stationarity led us to use only time slices where there was no secular trend in the data.) The CV was related to several other variables that indicate rhythmicity. 1) Rhythmicity index (Fig. 5A), which consists of the normalized power amplitude of the fundamental frequency peak (peak amplitude/baseline amplitude), was inversely related to the CV of interval durations. The two variables are intrinsically related in a reciprocal manner, because a low CV means that adjacent interval durations are close in value, which results in stronger rhythmicity as indicated in the autocorrelation and the autospectrum. 2) Number of autospectral peaks (fundamental plus harmonics; Fig. 5C) was also inversely related to CV, since strength of rhythmicity is reflected in the Fourier transform as number of harmonics. 3) Peak spectral frequency (Fig. 5D) had a more dispersed relation to CV, even though there was still a significant tendency for an inverse relation between frequency and CV values. This observation indicates that neurons with similar spectral frequencies may differ somewhat in their CVs.

The cumulative distribution of rhythmicity index versus CV values (Fig. 5B) had a shape that was neither linear nor sigmoidal. However, there seemed to be an inflection point near CV = 12% that suggested a separation of the curve into two segments, indicating existence of distinct ranges having lower or higher CV values. To evaluate this apparent separation, we computed and plotted (Fig. 5B) two linear regression lines, using values of the index in different ranges of CV: 1) CV values ≤12% (left line) or 2) CV value >12% (right line). Comparison of the two regression lines showed that the slope of the left line was 8.70 times the slope of the right line. Therefore we chose a CV of 12% (indicated by vertical lines in

FIG. 15. Effects of clonidine (1 µM) plus yohimbine (10 µM) perfusion on MP of an I-type neuron (top) during delivery of hyperpolarizing current pulses (bottom). The traces were obtained by playback of the signals into a chart recorder (spike amplitude being attenuated). A: (left) recording immediately before and after yohimbine perfusion onset (arrow) and (right) recording starts 1,050 s (arrow) after clonidine perfusion onset together with continuing yohimbine perfusion. Note that yohimbine did not block the usual inhibitory effect of clonidine (hyperpolarization, decrease of firing rate, and moderate increase of membrane resistance). B: effects of the hyperpolarizing pulse on membrane potential displayed on a faster timescale. Corresponding times of delivery of pulses in A and B: during yohimbine perfusion (*) and during clonidine plus yohimbine perfusion (**). Note the hyperpolarization and decreased firing rate in the presence of clonidine (**).
bulbospinal barosensitive neurons were found in the RVLM. Similar results were found in another study using in vivo rat aortic depressor nerve stimulation produced in those neurons a rhythmic hyperpolarization or depolarization. In addition, ring rhythms locked to the cardiac cycle, in the form of either tive neurons with membrane potential oscillations and spike ring neurons produced spontaneous postsynaptic potentials that increased in magnitude with greater hyperpolarization; thus some of these potentials could have been EPSPs. Only a few neurons in this group displayed anomalous rectification when the neuron was hyperpolarized by pulses of negative current. In addition, in this group we found that the current/voltage curve had an inward rectification.

Two major differences between the characterized R and I neurons were as follows. 1) There was a higher incidence of anomalous rectification in R neurons (14/18) than in I neurons (4/19); this was highly significant (P < 0.001 by the χ² test). 2) The mean autospectral frequency was greater for the R than for the I neurons (15.6 and 10.4 Hz, respectively); this was highly significant (P < 0.02 by the t-test). However the relation between frequency and CV was dispersed (Fig. 5C), i.e., neurons with similar CV values could have considerably different frequencies. Finally, there was no significant difference between R and I types for two other variables: action potential duration and resting membrane potential.

Since in the in vitro slice preparation there is no certain way of ascribing sympathetic-related function to the recorded RLVM neurons, it is necessary to compare their firing properties with those of RLVM neurons recorded in vivo. In a rat in vivo preparation (Zagon and Spyer 1996), intracellular recordings showed the presence in RLVM of bulbospinal barosensitive neurons with membrane potential oscillations and spike firing rhythms locked to the cardiac cycle, in the form of either a rhythmic hyperpolarization or depolarization. In addition, aortic depressor nerve stimulation produced in those neurons polysynaptic IPSPs as well as biphasic EPSP-IPSP sequences. Similar results were found in another study using in vivo rat preparations (Granata 1995; Granata and Kitai 1992), where bulbospinal barosensitive neurons were found in the RVLM that were designated as type I and type II, having electrophysiological characteristics that were similar to those of the I and R type neurons, respectively, of the present study. Of particular interest was the observation that some type II neurons had a very regular firing patterns during a period of reduced baroreceptor input (hypotension), but when normotension was restored the discharges became modulated in synchrony with the cardiac cycle (Figs. 7A and 9A in Granata and Kitai 1992). In that study, we analyzed 8 type II neurons and 17 type I neurons. We also analyzed 12 bulbospinal neurons (Fig. 5 in Granata and Kitai 1992) with a very regular discharge that had no temporal relation to the cardiac cycle. Thus in both in vitro and in vivo preparations there was heterogeneity of neuronal discharge types.

Another group of investigators (Lipski et al. 1996), using intracellular recordings in the in vivo rat preparation, has reported conflicting results: they could not find regularly firing bulbospinal barosensitive neurons. This negative result could have been due to technical deficiencies, since R-type neurons tend to be smaller than I-type neurons, as reported by Oshima et al. (2000), for the brain stem/spinal cord preparation. The small-sized neurons in the study of Lipski et al. (1996) could have been preferentially damaged, because even some of the larger (I-type) neurons in that study showed signs of damage (Fig. 8C in Lipski et al. 1996), thus indicating that the technique used was not suitable for penetration of the smaller R-type neurons in the RVLM.

Our positive results show that there is a significant population of R-type neurons in the RVLM of both in vivo preparations (Granata and Kitai 1992) and slice preparations (present study). Confirming evidence has been supplied in a recent study by Oshima et al. (2000), who used a whole cell patch-clamp technique in the neonatal rat brain stem/spinal cord preparation, which preserves the sympathetic neural network(s). They demonstrated the presence in the RVLM of two types of spontaneously active neurons (regularly and irregularly firing) as well as of silent neurons; these neuronal types resemble those of the present study.

At present we are not in a position to provide a detailed explanation of the mechanisms of generation of tonic sympathoexcitatory activity in RVLM presympathetic neurons. A well-known hypothesis is that sympathetic activity is generated by neuronal networks in the medulla (Barman and Gebber 2000). Another hypothesis is that sympathetic activity is generated by pacemaker-like neurons in the RVLM (Sun et al. 1988a). Our own data suggest that a more neutral term, such as "endogenous oscillators," be used for this population. We might speculate that the I neurons are more involved in synaptically mediated network interactions, whereas the R neurons function to provide background excitation to the network(s). Moreover, since both the R and I groups are found in the RVLM, it seems reasonable that interactions between the two populations may promote generation of sympathetic tone, as was proposed in earlier publications (Granata 1995; Granata and Kitai 1992).

Our quantitative criteria for regularity of firing may be useful for evaluation of possible interactions between different groups of neurons. 1) Although we distinguish between R and I firing by means of the location of the inflection point in the CV distribution (≤ 12% vs. >12%, respectively), there was a range of values around this point, indicating a possible transi-
When a neuron was nearly silent (e.g., as in Fig. 12D), due to depolarization, increase of mean rhythmicity. However, six neurons showed no excitatory response that was not followed by a depressant response. It is of interest to note that only I-type neurons responded with an early depolarization preceding the depression.

It is possible to speculate that some excitatory responses could be produced by disinhibition, due to activation of α2-adrenergic inhibitory presynaptic receptors on terminals mediating inhibitory responses of RVLM neurons. In support of this idea, the α2A-adrenergic receptor subtype has been localized in the RVLM on presynaptic terminals of noncatecholaminergic cells providing inhibitory inputs to neurons in this area (Milner et al. 1999).

The lack of effects of clonidine (at concentrations close to those in the present study) on putative sympathoexcitatory RVLM pacemaker-like neurons in vitro, as reported by Sun and Guyenet (1990), is in clear discrepancy with the present results, both with respect to depression and excitation. Furthermore, investigators of that group published opposing results regarding responses to clonidine shown by RVLM barosensitive bulbospinal neurons recorded extracellularly in vivo: (1) response by very few neurons (Sun and Guyenet 1986); (2) response only by a subpopulation (Allen and Guyenet 1993); and (3) inhibitory response by almost all neurons, including the C1-adrenergic group (Schreihofer and Guyenet 2000). In the last cited study, intravenous application of clonidine produced an initial short latency inhibition of sympathetic nerve and RVLM neuron activity; but according to the authors, this was a result of baroreceptor activation by the transient blood pressure rise due to peripheral action on arterial smooth muscle. Thereafter, there was a longer lasting inhibitory effect comparable to that observed in the present study. Furthermore, in the cited study (Schreihofer and Guyenet 2000), the limitation of the juxtacellular marking technique makes it uncertain that the C1 neurons were the targets of clonidine.

In the present study, the depressant effect of clonidine on the R-type RVLM neurons tested was completely blocked by the nonimidazoline α2-adrenergic antagonist rauwolscine. However, rauwolscine alone elicited depolarization and increase of firing. This excitatory effect of rauwolscine could be the result of blocking an inhibitory effect of endogenous catecholamines released from deafferentated terminals. Furthermore, clonidine in the presence of rauwolscine still produced a minor depolarization. Considering that the predominant depressant effect of clonidine on R-type neurons was due to maintained depolarization, it may be suggested that this remaining excitatory effect of clonidine could be the result of activating a different type of receptor. Hence, the maintained depolarization elicited by clonidine on the group of neurons characterized in the present study could be mediated by more than one type of receptor.

However, for one I-type neuron, rauwolscine failed to block the inhibitory effect of clonidine produced by hyperpolarization that followed the initial depolarization/excitation.

Furthermore, the antagonist yohimbine failed to block the actions of clonidine on two additional I-type neurons tested. Yohimbine, like rauwolscine, is a rauwolscia alkaloid family derivative and a very effective blocker of α2 adrenergic receptors with very low affinity for imidazoline receptors (Ernsberger et al. 1995; Harrison et al. 1991; Hieble and Kolpak 1993; Timmernans et al. 1981). Moreover, these agents are also potent antagonists of 5-HT1a receptors (Winter and Rabin 1992). The reason for the discrepancy between the effects of these two antagonists on R-type and I-type neurons is not apparent. We can speculate that the lack of blocking effect by yohimbine and rauwolscine is due to the inhibitory effects of clonidine on at least some I-type neurons being mediated by another subtype of α2-adrenergic receptors or perhaps by other types of receptor.

There is compelling evidence to support the idea that the central hypotensive effect of clonidine is mediated by stimulation of α2-adrenergic receptors, probably in sympathetic...
neurons of the RVLM. Experiments involving gene substitution in the mouse (Link et al. 1996; MacMillan et al.1996) demonstrated that the substitution of only one amino acid of the α2A-adrenergic receptor subtype produced a strain of mice with a dramatic downregulation in expression of α2A-adrenergic subtype receptors. These mutated mice lacked the hypertensive response to systemic injections of α2-adrenergic agonists, including clonidine. In addition, it is well established that α2-adrenergic receptors are present in high-density in the RVLM (Unnerstall et al. 1984).

In summary, we used quantitative criteria (based on frequency- and time-domain analysis) to designate regular or irregular spontaneously firing neurons intracellularly recorded in vitro in the RVLM. The α2-adrenergic agonist clonidine elicited a strong predominantly inhibitory effect on both populations of recorded neurons (21/26; 81%). However, the mechanism of action to achieve the final inhibition varied among different subgroups.

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