

# **Electrophysiological and Anatomical Characterization of Barosensitive Neurons in the Rostral Ventrolateral Medulla**

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## **I. Introduction**

The rostral ventrolateral medulla (RVLM) is an area in the ventrolateral segment of the medullary reticular formation that contains a particular population of neurons involved in the regulation of cardiovascular, respiratory, and nociceptive functions. The anatomical definition of the RVLM has been a matter of discussion, in part because the term has been used to define approximately similar medullary areas in different species; furthermore, in some studies the term has been used to define a functional area in this portion of the medulla [e.g., the vasopressor area in the RVLM (1-3)] that involves different anatomically identifiable nuclei, as, for instance, the paragigantocellular nucleus (4) and a more lateral portion called the retrofacial nucleus in the cat (5). In any case, definition of the RVLM on the basis of its anatomical cytoarchitectonic divisions has been difficult, even within the same genus (4,6,7). Nonetheless, the RVLM in the rat can be clearly defined inside the boundaries of an area limited dorsally by the nucleus ambiguus, which in its more rostral part is also called the retrofacial nucleus (8), and by the gigantocellular reticular nucleus; ventrally, the RVLM extends to the ventral medullary surface. The rostral limit

of the RVLM is the facial nucleus, and the caudal one is the lateral reticular nucleus. Mediolaterally, the RVLM extends from the spinal trigeminal tract and spinal trigeminal nucleus (pars oralis) to an area containing a group of neurons immunoreactive to serotonin and substance P. In the ventral aspect (9) and more dorsally, the RVLM extends to the nucleus gigantocellularis pars ventralis.

The RVLM is interconnected with different areas in the brain and the spinal cord directly related to autonomic control. The mediocaudal nucleus of the solitary tract (NTS) at the level of the primary afferents from baro- and mechanoreceptors (10,11) densely innervates the RVLM (12,13); in turn, the RVLM innervates similar areas of the NTS. A monosynaptic connection between the NTS and the RVLM was first postulated as the main intramedullary pathway of the baro- and mechanoreceptor reflexes (14). In contrast, other reports support the idea that neurons located in the caudal extension of the ventrolateral medulla, the so-called caudal ventrolateral medulla (CVLM), could serve as a relay station between the NTS and the RVLM (15,16). However, when bilateral electrolytic lesions are placed in the CVLM in the rat or the rabbit, the vaso-depressor responses elicited by electrical stimulation of the aortic depressor nerve or the central end of the vagus nerve are preserved (17,18). More recently, different reports have demonstrated that spontaneously active CVLM neurons were antidromically activated from the RVLM and excited by baroreceptor activation and by mediocaudal-NTS stimulation in the rat (19), whereas in the rabbit, these CVLM-RVLM neurons were excited by stimulation of the aortic depressor nerves and by inputs from the carotid sinus baroreceptors (20).

The central pathway of the sympathoinhibitory responses elicited by stimulation of visceral mechanoreceptor fibers in the vagus nerve is also controversial. The presence of interneurons receiving inputs from the NTS and projecting to the RVLM has been supported by some experimental evidence (21) and denied by others (22). Nevertheless, it is now accepted that both baroreceptor and visceral mechanoreceptor reflexes inhibit RVLM neurons with projections to the sympathetic preganglionic neurons in the intermediolateral column in the spinal cord, most probably through a relay station either in the CVLM or somewhere in the ventrolateral medulla (23,24).

On the other hand, it is still possible to speculate that the monosynaptic pathway between the mediocaudal-NTS and the RVLM conveys cardiovascular and pulmonary information, although its precise nature is still unknown.

The area postrema also innervates the RVLM (12,25,26); some studies in the rabbit suggest that noradrenergic neurons in the area postrema could contribute to this innervation (27), which could transmit information triggered by humoral factors unable to cross the blood-brain barrier.

The RVLM is also reciprocally connected with the lateral subdivisions of the parabrachial complex in both the rat and the cat (28,29). In addition, other studies have reported that the RVLM receives projections from, and in some cases is reciprocally connected with, the Kölliker-Fuse nucleus, the ambiguus

complex, the contralateral RVLM, the nucleus raphe magnus and raphe obscurus, the central gray area, the paraventricular nucleus, the lateral hypothalamic area, the caudolateral bed nucleus stria terminalis, and the zona incerta (26,29-33). Although the aforementioned areas are all related to autonomic control, the specific function of the connections or interconnections with the RVLM is still unknown.

The RVLM has been the focus of many investigations because of its critical role in regulating the vasomotor sympathetic outflow. Different studies have demonstrated that electrolytic lesions of the RVLM (34-36), application of different inhibitory agents or neurotransmitters into an area of the ventral medullary surface overlaying the RVLM (37-41), or reducing the temperature of this medullary surface (42) causes a profound decrease in arterial pressure similar to that produced by spinal cord transection. Conversely, electrical or chemical stimulation of this area results in an important increase in arterial pressure and heart rate (7,39,43,44). The possibility that neurons located in the RVLM could tonically excite sympathetic preganglionic vasomotor neurons in the spinal cord and then play the role of a "vasomotor center" has been strongly supported by anatomical studies demonstrating a direct projection from the RVLM to the intermediolateral and intermediomedial column in the spinal cord, innervating an area that corresponds to the distribution of sympathetic preganglionic neurons (7,44-46). Furthermore, anatomical studies using electron microscopy techniques combined with anterograde tract tracing of descending medullary projections and retrograde labeling of sympathoadrenal preganglionic neurons have demonstrated the existence of synaptic inputs from the RVLM to sympathetic preganglionic neurons (47). It is important to note that the distribution of bulbospinal neurons in the RVLM is restricted to the more rostral portion of this area; the vast majority of these neurons are distributed in an area approximately 800 to 1200  $\mu\text{m}$  rostral to the obex in the rat (48).

Nearly 50% of these bulbospinal neurons in the RVLM are immunoreactive to phenylethanolamine *N*-methyltransferase (PNMT), the enzyme that catalyzes the conversion of noradrenaline to adrenaline; consequently, these neurons are identical to the adrenergic group described by Höckfelt et al. (49) as the C1 adrenergic group. Because of the good correspondence between the location of the C1 adrenergic group, the bulbospinal neurons, and the medullary area critically involved in maintaining normal values of arterial pressure and heart rate, this subdivision of the RVLM has been called the C1 area (7, 18). In addition, synaptic contacts between adrenergic terminals immunoreactive to PNMT and identified sympathetic preganglionic neurons have been shown by electron microscopy. Since the RVLM is one of the more important sources of adrenaline to this part of the spinal cord, it has been assumed that C1 neurons contribute to the direct monosynaptic control of sympathetic preganglionic neurons (50). However, the possibility that these C1 adrenergic bulbospinal neurons could play the role of vasomotor neurons still poses an unresolved question. This point will be analyzed later in this study.

The critical "vasomotor" area (C1 area) involves only a *subdivision* of the nucleus paragigantocellularis lateralis in the rat (4,51); nonetheless, some investigators have called this area the "nucleus paragigantocellularis" (52). More accurately, Ross et al. (7) have anatomically defined this area of the reticular formation in the rat as the nucleus reticularis rostroventrolateralis. This area appears to be similar to the retrofacial nucleus in the cat described by Berman (8), and a seemingly similar area in the cat has been termed the "subretrofacial nucleus" by others (53).

The intermediolateral cell column also receives direct projections from the raphe pallidus, the raphe obscurus, the A5 noradrenaline-containing neurons in the ventral pons, the Kölliker-Fuse nucleus, the lateral hypothalamus, and the paraventricular nucleus in the hypothalamus. None of these pathways, however, has been demonstrated to play a role in vasomotor control.

Using extracellular recording techniques, different laboratories have identified a group of neurons in the RVLM that can be antidromically activated from the thoracic spinal cord with velocities of conduction below 10 m/s; these neurons were identified in the rat, rabbit, and cat (53-56). The recording sites, although not necessarily the neuronal location, appear to correspond to the nucleus reticularis rostroventrolateralis in the RVLM of the rat (7). The recorded neurons were spontaneously active, with a high frequency of discharge that was synchronized to the arterial pulse wave; and they were inhibited by an increase in systemic arterial pressure. In addition, the spontaneous discharge was also related to the lumbar and splanchnic sympathetic nerve discharge (55, 57). Therefore, these neurons demonstrated barosensitive properties and were considered to be components of the tonic bulbospinal sympathoexcitatory pathway.

Although some electrophysiological evidence suggests that these tonic bulbospinal neurons with barosensitive characteristics in the RVLM project to the intermediolateral column in the spinal cord (55), whether they send monosynaptic potentials to the sympathetic preganglionic neurons is still unknown.

More recent investigations have proposed that an area in the RVLM situated more medially than the classically defined "vasomotor" area—and hence also medially from the rostral pole of the C1 adrenergic neurons—could have a sympathoexcitatory function similar to that of the lateral part (58,59). Furthermore, it has been proposed that neurons in this area acting separately from the lateral counterpart could also contribute to vasomotor control, although affecting different peripheral vascular beds from those controlled by the lateral part (59-62). This medial part contains neurons that are immunoreactive to serotonin and that project to the lateral horn in the spinal cord (58,63). However, electrophysiological studies using extracellular recording techniques have not recognized barosensitive neurons with different characteristics in the RVLM, although, according to the location of the recording sites, neurons in both subdivisions of the RVLM were supposedly recorded from those studies (54,64).

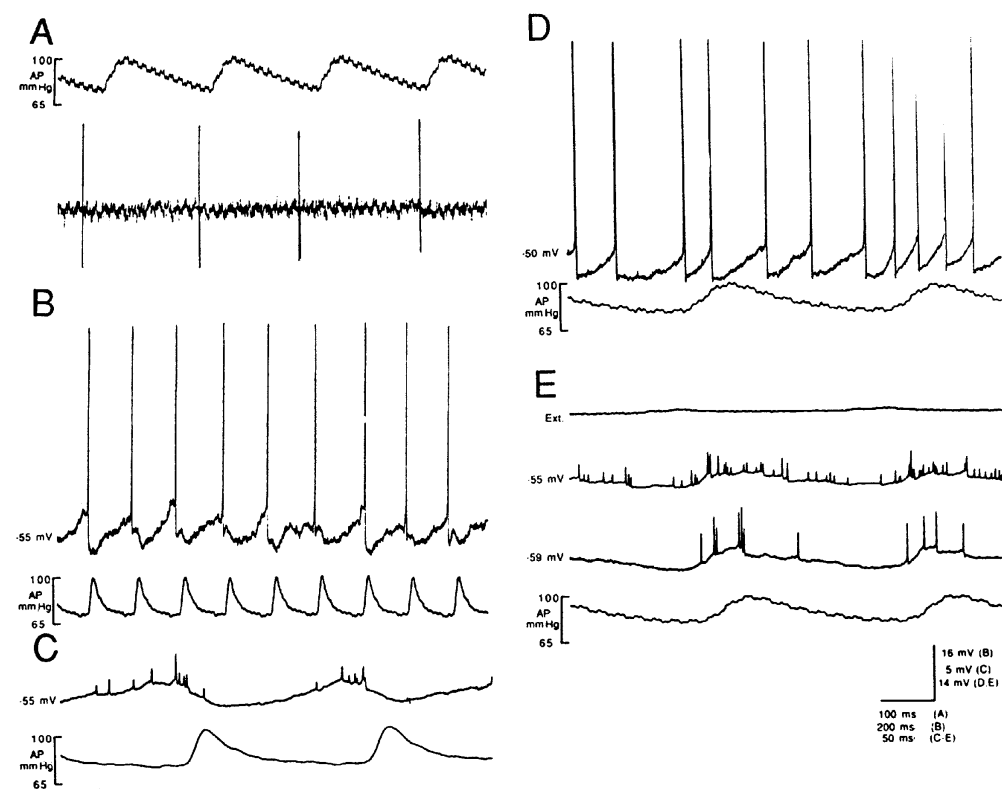
We have to take into consideration at this point the technical limitations inherent in the extracellular recording technique, which cannot determine either the precise localization of the recorded neurons or the synaptic inputs received by these neurons during the cardiac cycle.

We have recently developed a technique for recording intracellular potentials from RVLM neurons *in vivo* (65). In this paper, we discuss the results of experiments aimed at characterizing RVLM bulbospinal neurons with barosensitive properties. The recorded neurons were labeled by intracellular injections of biocytin; this technique was combined with immunocytochemical identification of PNMT to determine whether the electrophysiologically characterized neurons were the same as the C1 adrenergic group. Finally, we have also developed an *in vitro* intracellular recording technique that has been used to identify similar RVLM neurons previously characterized *in vivo*.

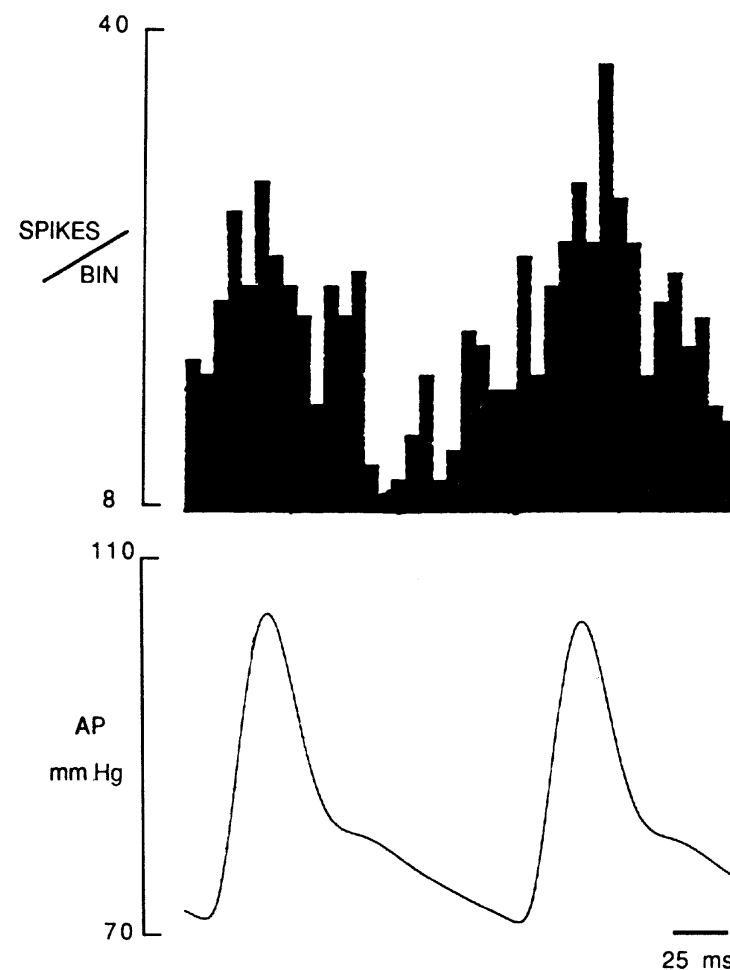
## II. Electrophysiological Identification of RVLM Neurons with Baroreceptor-Related Activity and Possible Involvement in Vasomotor Control

Several laboratories using an *in vivo* extracellular recording technique have identified a group of tonically active RVLM neurons antidromically activated from the sympathetic spinal cord and modulated by baroreceptor inputs (54-56,64). However, due to the technical limitations inherent in the extracellular recording technique, it was not possible to directly investigate the nature of these baroreceptor inputs or the transmitter produced by the bulbospinal neurons that receive these baroreceptor inputs.

We have developed a technique to record intracellular potentials *in vivo* and have investigated the RVLM for spontaneously active neurons with neuronal activity synchronized to the cardiac cycle. Our studies were conducted in anesthetized, paralyzed, ventilated rats (65). We investigated first at the extracellular level a group of neurons in the rostral portion of the RVLM with spontaneous action potentials occurring about every cardiac cycle, as demonstrated by simultaneous recording of the pulsatile arterial pressure and the extracellular recording (Fig. 1A). Such neurons could correspond to those described by other investigators using extracellular recording techniques (55,57). Other neurons located in the same area did not show such a clear synchronization at the extracellular level. However, simultaneous pulse-triggered histograms of the neuronal activity and averages of the pulsatile arterial pressure demonstrated a clear cardiac modulation of neuronal discharge with a conspicuous peak in the neuronal discharge in each cardiac cycle (Fig. 2). Similar types of synchronization have been reported by other investigators using an extracellular recording technique to characterize barosensitive neurons in the RVLM in the rat and the cat (53, 55).



**Figure 1** Extracellular and intracellular recordings from different bulbospinal RVLM neurons with cardiac-related activity. (A) Digitized record of extracellular recording of a spontaneously active RVLM neuron and arterial pressure (AP) in mm Hg. (B) Intracellular recording of the same neuron as in panel A at stable resting membrane potential ( $-55$  mV). The neuron has recovered its pulse-related firing pattern observed in panel A. The digitized records of AP and membrane potential were simultaneously triggered by the pulsatile AP. (C) Simultaneous average of the neuronal membrane potential and pulsatile AP at resting values. The averages were triggered by the arterial pulse wave. Number of sweeps = 450. Sample period =  $50 \mu\text{s}$ . (D) Simultaneous digitized records of arterial pressure (AP) (mm Hg) and intracellular recording of a different neuron at stable membrane potential ( $-50$  mV) triggered by the arterial pulse wave. Note the increased frequency of discharge at the end of diastolic periods. (E) Simultaneous average of the AP in millimeters of mercury at resting values and the spontaneous neuronal membrane potential (upper middle trace) and the neuronal membrane potential held at  $-59$  mV (lower middle trace). The average of the extracellular (ext.) recording is illustrated in the uppermost trace. The averages were triggered by the arterial pulse wave. Same neuron as in panel D. Number of sweeps was 550 in all cases. Sample period =  $50 \mu\text{s}$ . (From Ref. 65.)



**Figure 2** Pulse-triggered histogram of RVLM neuron and simultaneous average of arterial pressure. Upper and lower traces show number of spikes occurring in each time bin and the corresponding arterial pulse, respectively. Sweeps = 400; bin width = 5 ms for histogram, 1 ms for average. (From A. R. Granata, unpublished results.)

Neurons with the aforementioned characteristics were selected for intracellular analysis. During intracellular recording, some of them demonstrated a pattern of discharge similar to that found during extracellular recording; namely, spontaneous depolarizing and action potentials at practically every cardiac cycle (Fig. 1B). The simultaneous averages of pulsatile arterial pressure and neuronal membrane potential also demonstrated a clear cardiac-related rhythmic activity in this type of RVLM neuron (Fig. 1C); the average depolarization of the membrane potential correlated with the period of the pulsatile arterial pressure.

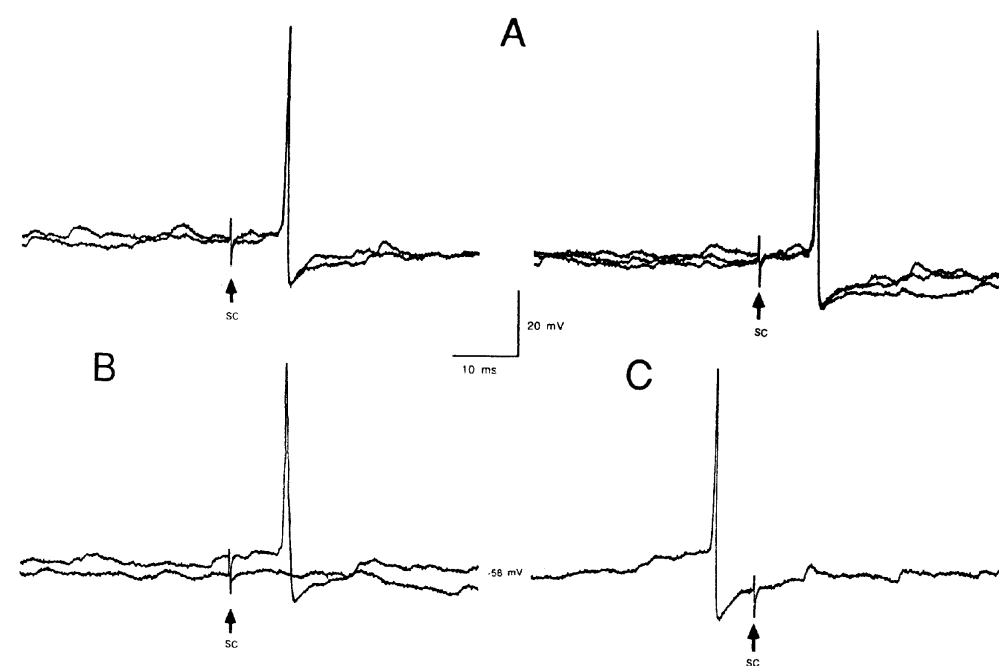
In another neuron, the cardiac-related rhythmic activity was demonstrated by a consistently faster rate of discharge at the end of the diastolic/beginning of the systolic period during spontaneous membrane potential (Fig. 1D). In these neurons, the simultaneous average of the neuronal membrane potential and pulsatile arterial pressure also demonstrated depolarization potentials occurring at the end of the diastolic/beginning of the systolic period (Fig. 1E). These potentials increased in magnitude when the neuron was hyperpolarized by passing negative current through the recording electrode (Fig. 1E). Conversely, when the neuron was depolarized, the depolarization potential decreased in magnitude. Therefore, the potentials locked to the cardiac cycle were considered excitatory postsynaptic potentials (EPSP) because of the changes in magnitude observed during hyperpolarization and depolarization.

Support for the view that these neurons with pulse-modulated activity send projections to the intermediolateral column in the spinal cord comes from electrophysiological experiments (65). We have observed that barosensitive RVLM neurons can be antidromically driven by electrical stimulation of the intermediolateral column of the ipsilateral thoracic spinal cord at the T2-T3 level (Fig. 3A to C).

The calculated conduction velocities of the descending axons from RVLM neurons characterized as barosensitive ranged from 1.2 m/s to 11.0 m/s. Other investigators have reported a second type of RVLM bulbospinal neuron with pulse-modulated activity but with slower axonal velocities of conduction. Yet in one case, these neurons, although located in the RVLM, were silent and thus incompatible with the role of tonic sympathetic nerve activity generator (55); whereas in the other case, the slowly conducting neurons were spontaneously active but located in the more medial aspect of this tonic vasomotor area—the nucleus paragigantocellularis lateralis (57).

Under our experimental conditions, we did not find spontaneously active RVLM neurons with both barosensitive properties and slow axonal velocities of conduction.

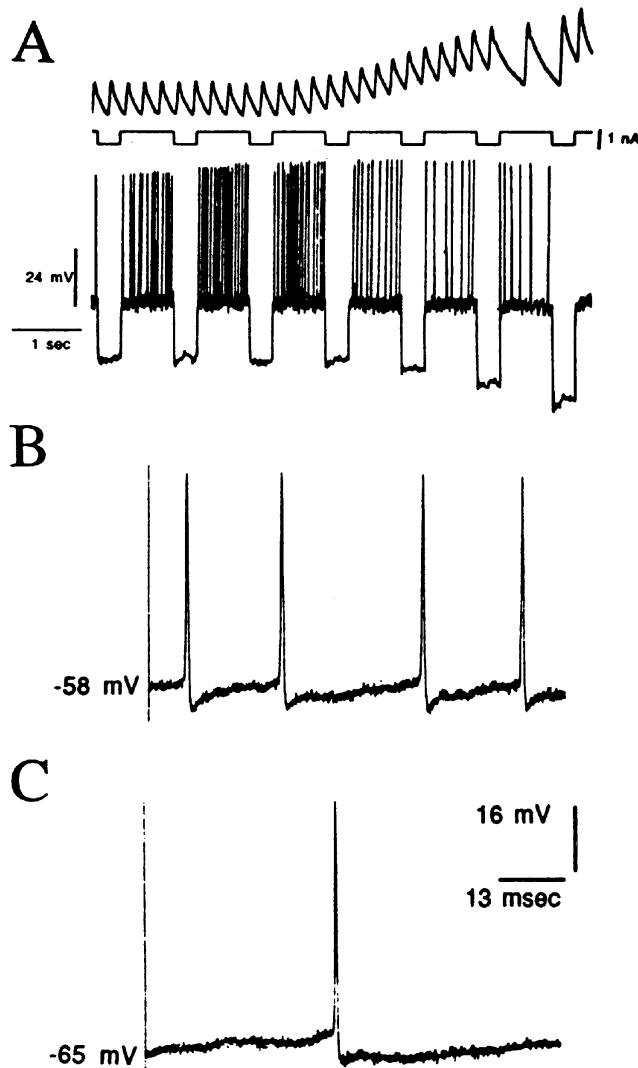
We noticed that the baroreceptor inputs were able to control the total activity of these RVLM neurons with EPSP synchronized to the cardiac cycle. The activation of the baroreceptor reflex, by increasing systemic arterial pressure with bolus intravenous injections of phenylephrine, produced hyperpolarization, a decrease in the firing frequency, and an increase in the membrane input resistance in barosensitive neurons with depolarizing potentials locked to the cardiac cycle (Fig. 4). In these experiments, the input impedance was calculated by deflections in the membrane potential evoked by constant pulses of negative current. On the other hand, the inactivation of the baroreceptor reflex by decreasing systemic arterial blood pressure by bolus intravenous injections of the peripheral vasodilator sodium nitroprusside elicited the opposite effect in these barosensitive neurons: specifically, depolarization and an increase in the firing frequency (65).



**Figure 3** Antidromic activation of an RVLM neuron with pulse-modulated activity following spinal cord stimulation. (A) Electrical stimulation of the IML in the ipsilateral spinal cord (arrow) during intracellular recording produced action potentials, which demonstrated constant latency. (B) At stimulation threshold ( $75 \mu\text{A}$ ) (arrow), spikes were all or none, and no underlying excitatory postsynaptic potentials were observed when the spike failed (the DC trace is shifted to improve visualization). (C) A spontaneous spike collided with the antidromic elicited action potential at  $2 \times$  threshold of stimulation (arrow). (From Ref. 65.)

In consequence, these studies demonstrate the participation in the baroreceptor reflex of barosensitive neurons with EPSP locked to the cardiac cycle. Furthermore, they also indicate that disfacilitation is responsible for the hyperpolarization accompanied by a decrease in the firing frequency and an increase in the membrane input resistance observed following baroreceptor activation. Since it is well known that tonic sympathetic nerve activity is generated in the brainstem (66), this disfacilitation could originate in the inhibition of tonic excitatory inputs produced by other neurons in the brainstem.

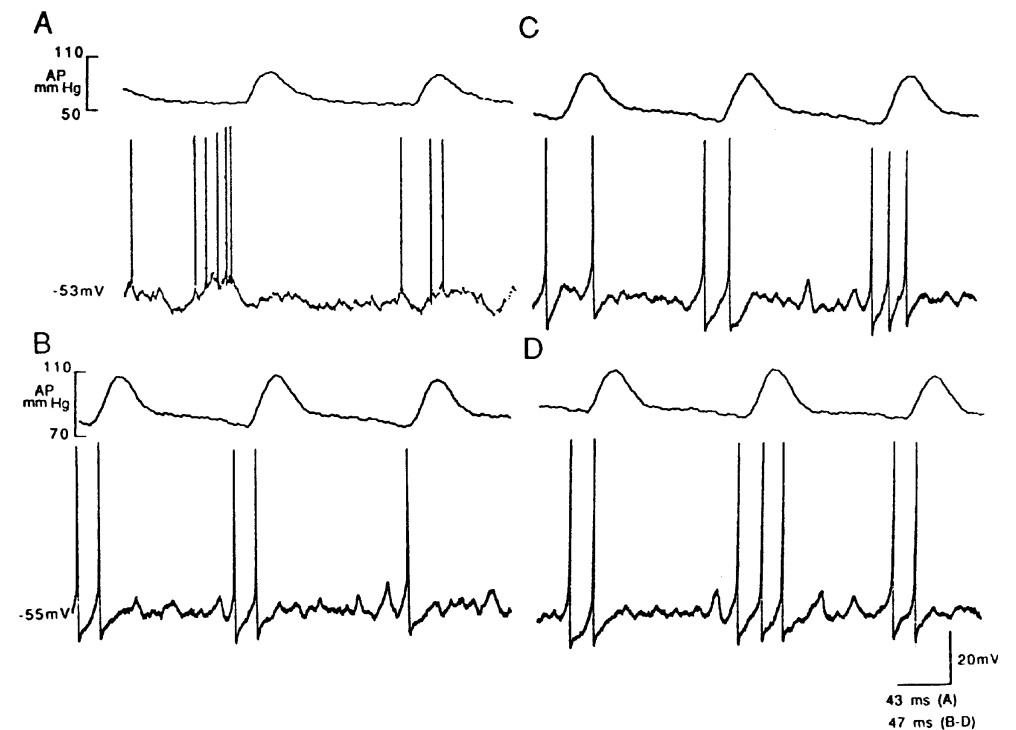
In addition, these tonic excitatory inputs could be responsible for another characteristic found in the barosensitive RVLM neurons with EPSP locked to the cardiac cycle: the presence of an important ongoing and random synaptic activity observed at resting values of arterial pressure, heart rate, and neuronal membrane potential (Figs. 3 and 5).



**Figure 4** Inhibition of a bulbospinal RVLM neuron by phenylephrine-produced increases in systemic arterial pressure. (A) Intravenous administration of a bolus injection of phenylephrine during intracellular recording from a bulbospinal neuron in the RVLM elicited: (a) a decrease in the firing frequency; (b) an increase in the input resistance, as demonstrated by the increased amplitude of hyperpolarizing voltage deflections in response to constant-amplitude inward current pulses; and (c) hyperpolarization of the neuronal membrane potential, which can be clearly seen when the gain of the amplifier is increased: B = control; C = hyperpolarization during baroreflex activation. Note that the membrane input resistance increased in proportion to changes in arterial pressure and preceded the reflex bradycardic response. From top to bottom in panel A, systemic arterial pressure (mm Hg), control current injections, and intracellular recording (membrane potential  $-49\text{mV}$ ). All spikes are attenuated in size. (From A.R. Granata, unpublished results.)

We do not know the location of these tonic excitatory neurons in the rat. However, other investigators working in the cat have postulated that sympathoexcitatory neurons located in the nucleus reticularis parvocellularis and nucleus reticularis ventralis in the lateral tegmental field drive RVLM-spinal neurons by a direct pathway from these nuclei in the lateral tegmental field to the RVLM (64,67-69).

Another characteristic that distinguishes these barosensitive neurons with EPSP locked to the cardiac cycle is that, besides firing in a single spiking mode, they have been observed to fire in a bursting pattern, with a burst occurring at the end of the diastolic/beginning of the systolic period; on several occasions the burst rode on a depolarizing potential (Fig. 5). At normal values of arterial pressure and heart rate, with the animal in a paralyzed condition and under urethane anesthesia, the bursts are composed of an arrangement of 2 to 5 spikes. Furthermore, we observed that some of these barosensitive neurons firing in a single-spiking mode switched to a bursting mode. This change in the firing mode could be the result of a relatively prolonged period of excitation, so the



**Figure 5** Spontaneously occurring burst intracellularly recorded from two barosensitive RVLM neurons. In panel A, the synchronized bursts were observed to ride on a depolarizing potential. In panels B-D (in a different neuron), the bursts or sometimes single spikes were also synchronized to the cardiac cycle. Note the important ongoing activity in this type of neuron. (From Granata, unpublished results.)

excitatory inputs received by these neurons could play even an additional role by producing a summation of spikes in the terminals of these descending neurons in the intermediolateral cell column. The summation of spikes could result in an extensive release of neurotransmitter and, hence, in large increases in postsynaptic efficacy (70,71). Therefore, the physiological role played by these barosensitive neurons could differ from that of other RVLM neurons with barosensitive properties.

Based on the aforementioned characteristics, which can be clearly distinguished from those of other RVLM-spinal neurons, we have classified these neurons as barosensitive type I neurons.

Attempts to identify and characterize barosensitive neurons in the RVLM according to the phasic baroreceptor modulatory inputs received by these neurons during each rise in systolic arterial pressure have proved difficult for some investigators lacking sufficient experience in intracellular recording from small rodents. It is very possible that the presence of conspicuous mechanical artifacts related to arterial pulsation have prevented a clear identification of these baroreceptors' inputs on barosensitive RVLM neurons. It is beyond the scope of this paper to discuss the reasons and origins of such mechanical artifacts as could appear in the hands of other investigators due to different technical deficiencies. However, we will discuss the very careful analysis followed in our investigation to eliminate the presence of prominent mechanical artifacts from our intracellular recording. First, at the end of the intracellular recording session, the electrode was withdrawn from inside the neuron to just outside it; a number of extracellular recordings were performed, and the average was calculated. We considered as valid only those intracellular recordings associated with flat averages of the corresponding extracellular control, because the flat extracellular average indicates the absence of important mechanical artifacts that could have interfered with the intracellular potentials. The possibility that flat extracellular averages could have been the result of a reduction in the recording electrode resistance due to removal of the resistance of the whole neuron by moving the electrode from the intracellular to the extracellular space is excluded. This is because the neuronal input resistance calculated as the slope of the regression line derived from the plot of the current injection versus the deflection in membrane potential ranged between 10 to 15 M $\Omega$  in these RVLM neurons; we induced similar changes in electrode resistance by passage of constant current during extracellular recording and were unable to detect fluctuations in membrane potentials locked to the cardiac cycle. Furthermore, the variability in resistance among the different electrodes used in these experiments was three to four times larger than the membrane input resistance of these neurons.

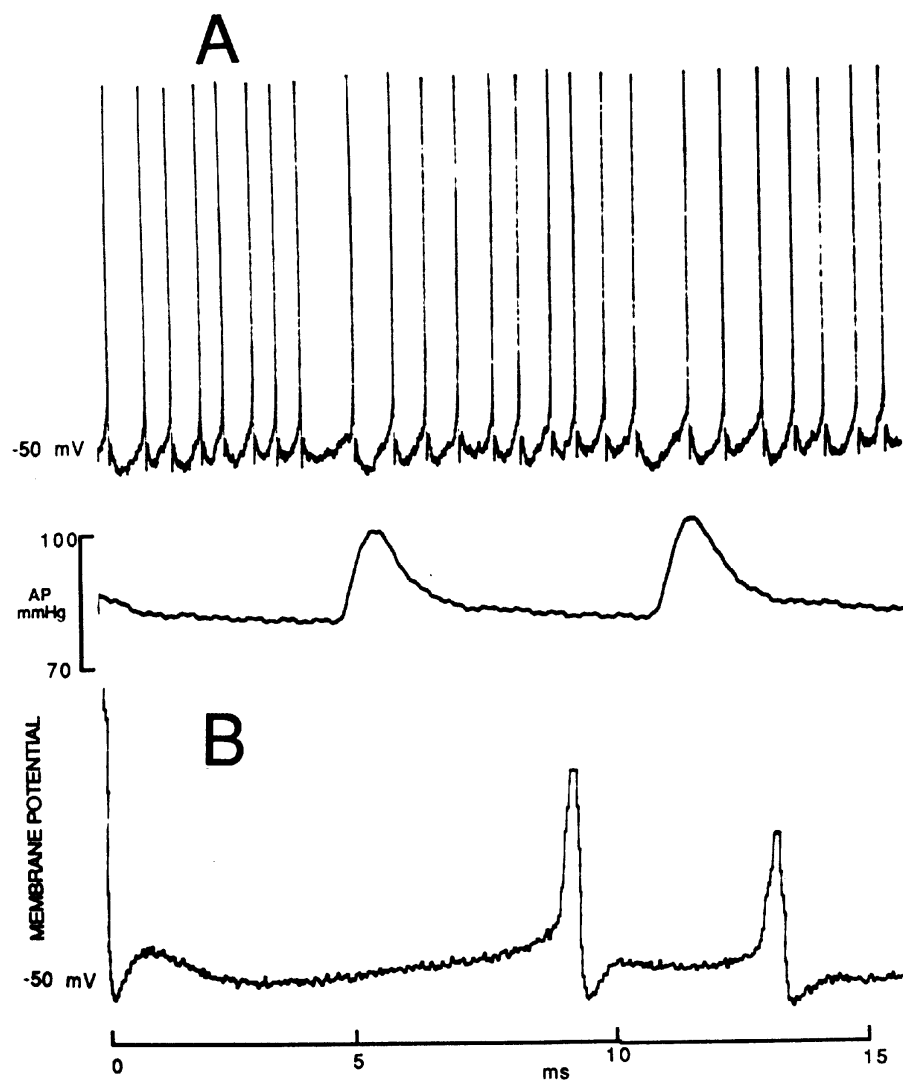
Second, we obtained intracellular potentials from a different type of neuron intermixed with barosensitive neurons in the RVLM. These different neurons can also be antidromically activated from the spinal cord; on the other hand,

they show a very regular pattern of discharge at normal levels of arterial pressure and a flat average of both the neuronal membrane potentials and extracellular recordings triggered by the arterial pressure pulse. We have never seen baroreceptor modulatory activity in this type of RVLM neuron when the recording conditions and the general physiological parameters of the rat were maintained strictly under the limits of normality. Therefore if, under normal recording conditions, mechanical artifacts were unavoidable although randomly present phenomena, some neurons with the characteristics already mentioned could have demonstrated mechanical artifacts resembling baroreceptor modulatory activity. Similarly, we consistently found that the average of the neuronal membrane potential triggered by arterial pressure pulse was flat in RVLM neurons with rhythmic activity synchronized to the respiratory cycle (65).

### III. Electrophysiological Characterization of RVLM Neurons with Inhibitory Postsynaptic Potentials (IPSP) Synchronized to the Cardiac Cycle

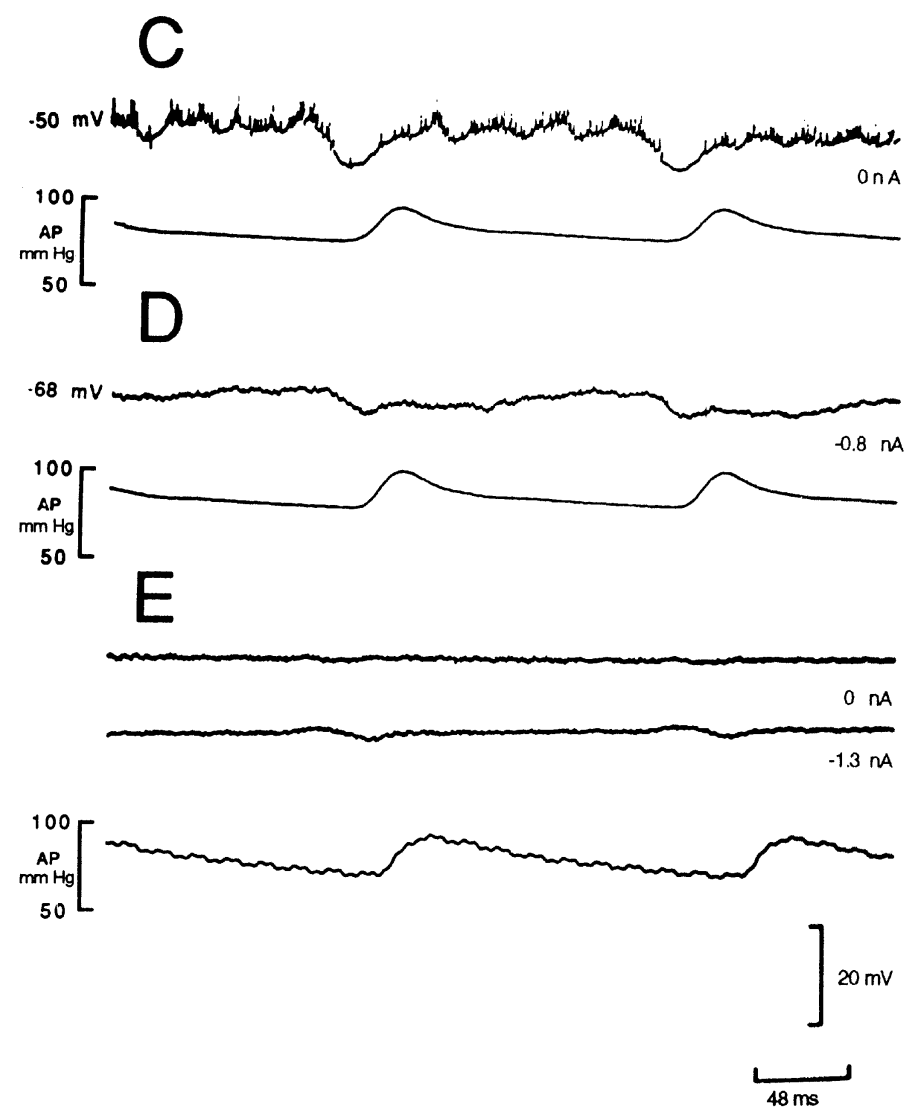
We have identified a different type of neuron in the RVLM on the basis of the pattern of discharge and responses to spinal cord stimulation. In this type of neuron, electrical stimulation of the intermediolateral column in the thoracic spinal cord evoked antidromic action potentials. Moreover, in some of these neurons, the antidromically evoked spike was preceded by a short-latency monosynaptic EPSP (65). On the other hand, the axonal conduction velocities of these bulbospinal neurons varied in a range similar to that observed in barosensitive type I neurons.

The neurons described in this group are spontaneously active, and the action potentials are produced by slow depolarizing potentials that drive the membrane potential to spike generation threshold. At spontaneous membrane potential and normal values of arterial pressure and heart rate, this type of neuron demonstrated a spontaneous firing pattern that included slower single spiking at the end of the diastolic/beginning of the systolic period, followed by single spikes firing at higher frequencies during the rest of the cardiac cycle (Fig. 6A). We observed more clearly this phasing pattern of discharge when the average of the membrane potential was triggered by spontaneous spikes (Fig. 6B). For the same neuron, at normal values of arterial pressure and heart rate, the post-R wave simultaneous average of the membrane potential and arterial pressure showed hyperpolarizing potentials occurring at the end of the diastolic/beginning of the systolic period (Fig. 6C). These hyperpolarizing potentials are inhibitory postsynaptic potentials (IPSP) because they decreased in magnitude when the neuron was hyperpolarized by passing negative current through the recording electrode (Fig. 6D). The control average of the extracellular recordings with the electrode still very close to the neuronal membrane and triggered

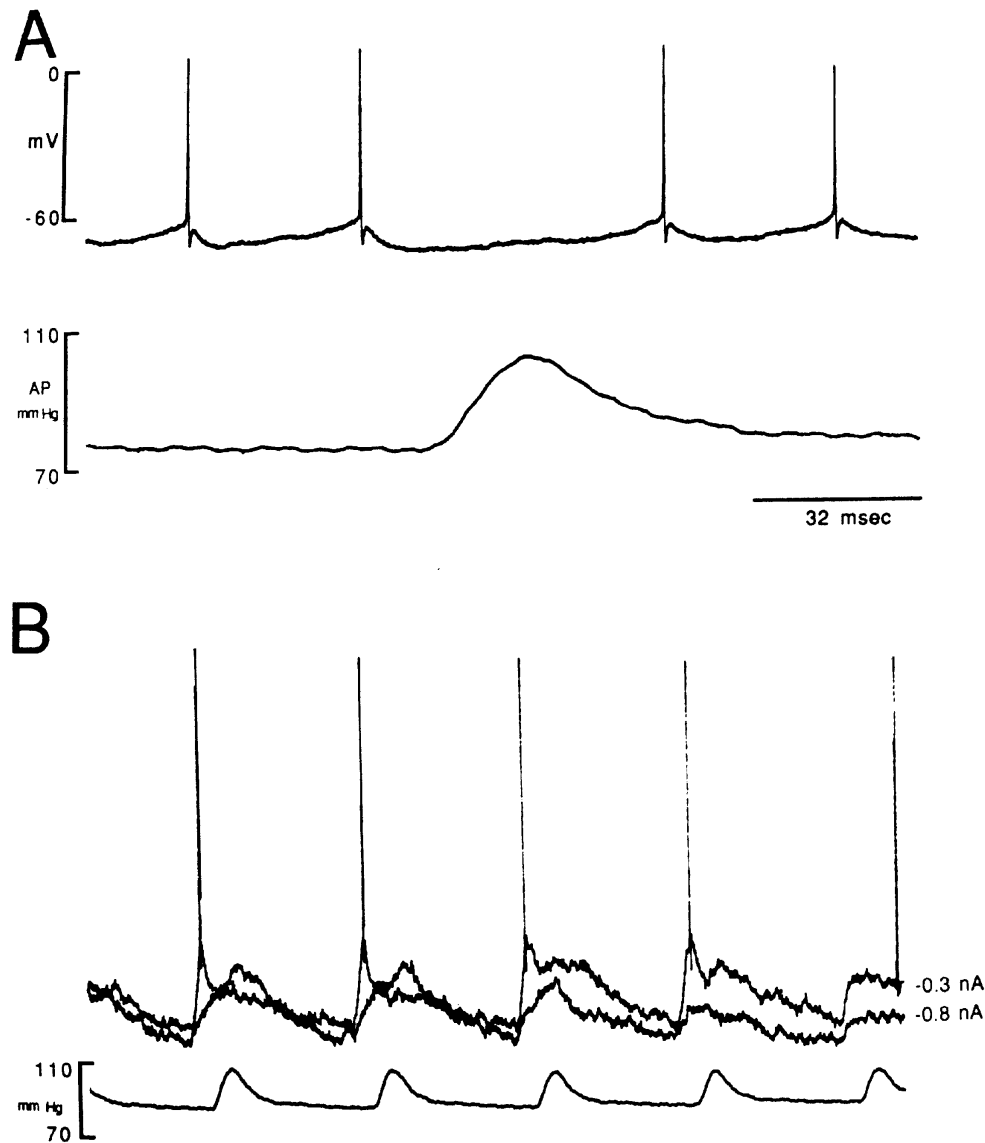


by the pulsatile arterial pressure was demonstrated to be flat (Fig. 6E). Furthermore, when the extracellular average was performed under constant injection of negative current through the recording electrode with intensities even higher than those used to drive the membrane potential during intracellular recording, only minor mechanical distortions were observed (Fig. 6E).

In these studies the IPSP were further characterized by injecting the neurons with chloride ions for several minutes. This was accomplished by passing negative current through the recording electrode filled with KCl. After the injection of chloride ions, we found that the hyperpolarization potential synchronized

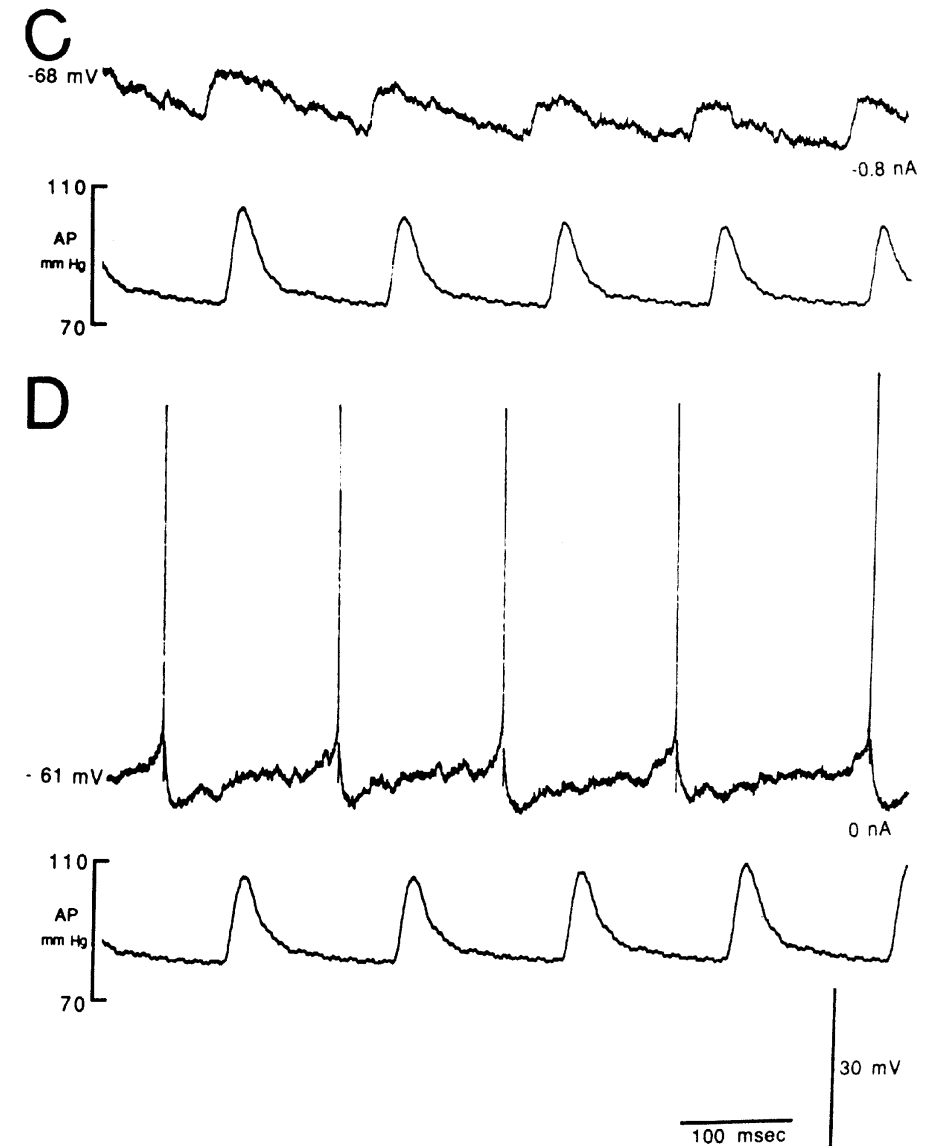


**Figure 6** Intracellular recording of a RVLm bulbospinal neuron with inhibitory baroreceptor inputs. (A) Simultaneous digitized records of arterial pressure (AP) in millimeters of mercury and intracellular recording at stable membrane potential ( $-50$  mV). The records were triggered by the arterial pulse wave. Note the decrease in the firing frequency at the end of the diastolic period. (B) Average of the resting neuronal membrane potential triggered by spontaneous spikes. Sweeps = 96; sample period =  $5 \mu\text{s}$ . (C) Simultaneous averages of the neuronal membrane potential ( $-50$  mV) and pulsatile AP in millimeters of mercury, triggered by the arterial pulse wave. Sweeps = 674; sample period =  $50 \mu\text{s}$ . (D) Averages similar to those illustrated in panel C, but now at a holding membrane potential of  $-68$  mV. (E) The simultaneous averages of AP and extracellular recordings at zero or  $-1.3$  nA constant current injection (upper and middle traces, respectively). The averages were triggered by the arterial pulse wave. Sweeps = 700; sample period =  $50 \mu\text{s}$ . Same neuron as in panels A through E. (From Ref. 65.)



to the cardiac cycle reversed in polarity (Fig. 7B). In addition, depending on the holding membrane potential, these reversed potentials that are synchronized with the cardiac cycle also produced action potentials at every cardiac cycle (Fig. 7D). These findings, therefore, indicate that the IPSP locked to the cardiac cycle are chloride-mediated IPSP.

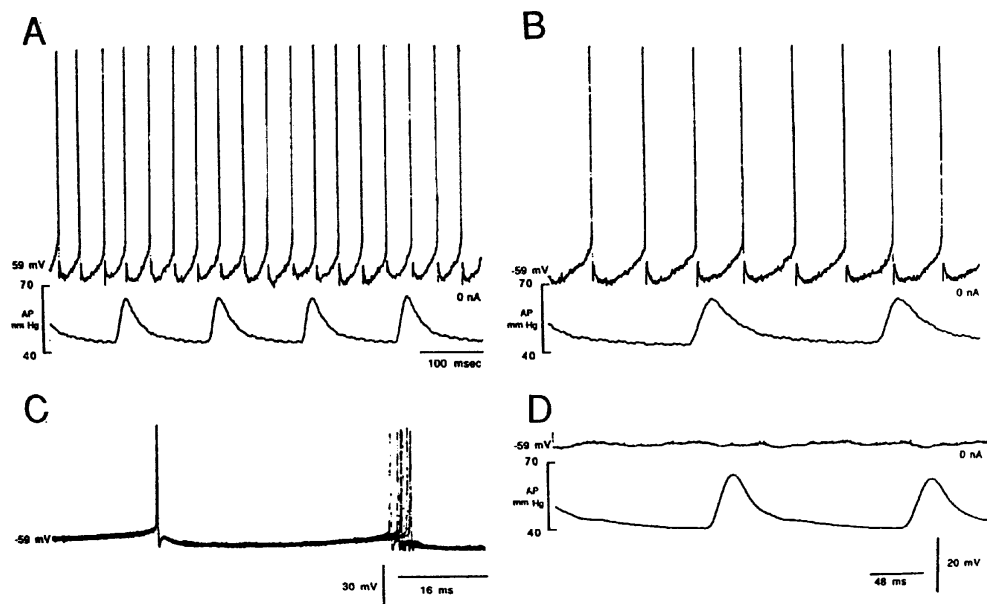
We discovered that the pattern of discharge of these neurons changed completely when systemic arterial pressure was constantly reduced by intravenous



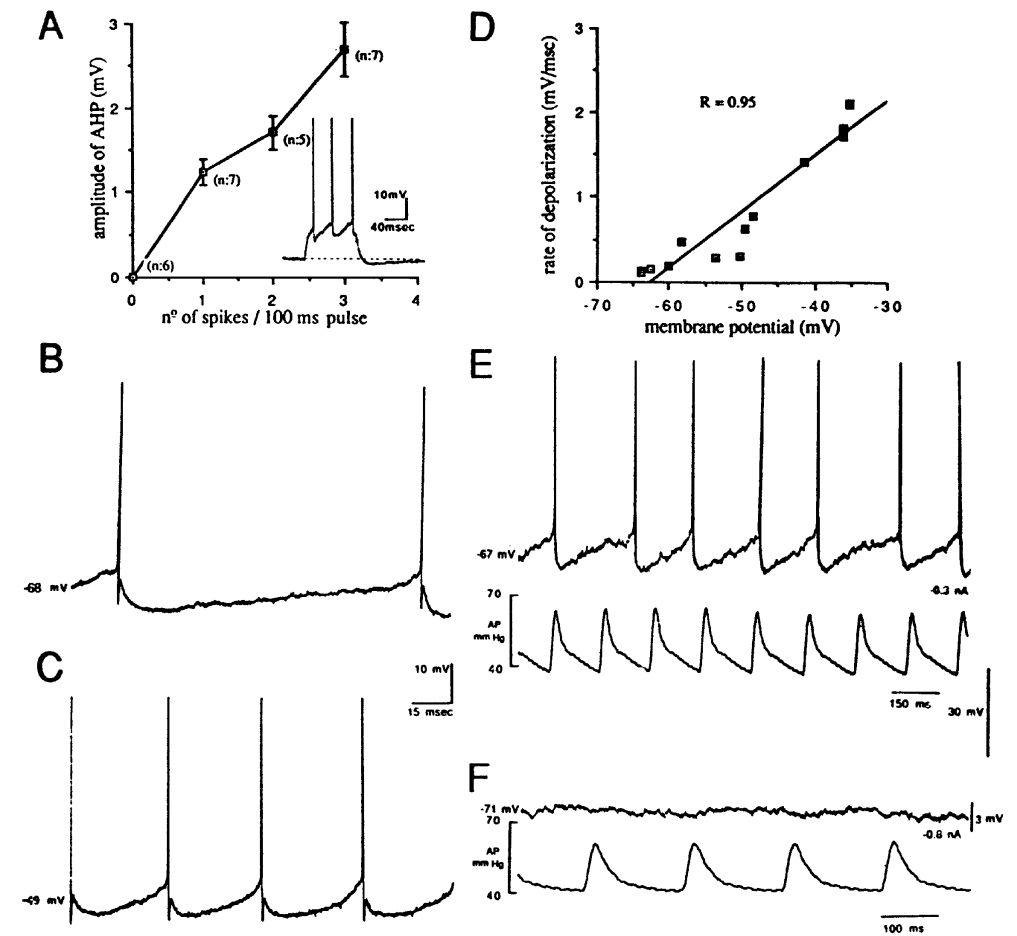
**Figure 7** Intracellular recordings of a RVLM bulbospinal neuron with inhibitory baroreceptor inputs during intracellular injection of negative current. (A) Simultaneous records of arterial pressure and intracellular recording, immediately after holding the membrane potential at more negative values. The records were triggered by the arterial pulse wave. (B) Similar records as in panel A (note different time base) after intracellular injections of negative current for several minutes and holding the membrane potential with  $-0.3$  nA or  $-0.8$  nA negative current (microelectrode filled with KCl). (C) Simultaneous records of arterial pressure and neuronal membrane potential under similar experimental conditions as in panel B, at a holding potential of  $-68$  mV. (D) Similar record as in panel (C), but at a membrane potential of  $-61$  mV. Same neuron as in panels A through D. (From Ref. 65.)

infusions of sodium nitroprusside. The firing pattern during hypotension became very regular, demonstrating a pacemakerlike activity (Fig. 8A and B). The regular firing pattern was also demonstrated by the unimodal distribution of action potentials perceived when the oscilloscope was triggered by spontaneous spikes (Fig. 8C). Furthermore, the IPSP locked to the cardiac cycle present at normal values of arterial pressure and heart rate vanished during hypotension. In consequence, during hypotension, the average of the neuronal membrane potential triggered by the pulsatile arterial pressure, either at spontaneous membrane potential or during hyperpolarization (accomplished by injecting a high-intensity negative current), was flat (Figs. 8D and 9F). In addition, the regular firing pattern observed during hypotension was lost when the neurons were hyperpolarized; nevertheless, the spikes were found unrelated to the arterial pulse (Fig. 9E).

These studies indicate that these RVLM bulbospinal neurons with baro-sensitive properties are inhibited by chloride-dependent IPSP entrained to the



**Figure 8** Intracellular recording of a RVLM bulbospinal neuron with inhibitory baroreceptor inputs during constant hypotension. (A) Simultaneous records of arterial pressure (AP) and intracellular recording at resting membrane potential during hypotension elicited by intravenous infusion of sodium nitroprusside. The records were triggered by the arterial pulse wave. Note the very regular firing pattern that took place when AP had been lowered. (B) Similar records as in panel A, but at different time base. (C) Seven superimposed oscilloscope traces triggered by spontaneous spikes. (D) Simultaneous averages of the neuronal membrane potential and pulsatile AP triggered by the arterial pulse wave during hypotension. Number of sweeps = 690; sample period = 50  $\mu$ s. Experimental conditions in panel A also applicable to panels B, C, and D. (From Ref. 65.)



**Figure 9** (A) Influence of the number of spikes elicited in a depolarizing current pulse on the amplitude of the afterhyperpolarization spike train. (B–D) Effects of changes in neuronal membrane potential on the rate of rise of the slow depolarization in RVLM neurons. (E–F) Effects of hyperpolarization and constant hypotension on a RVLM bulbospinal neuron with inhibitory baroreceptor inputs. (A) Intracellular injections of depolarizing current pulses (100 ms) with different intensities elicited to 0 to 3 spikes in RVLM neurons. At the end of the current pulse, an afterhyperpolarization was observed, as illustrated in a typical case in panel A. The amplitude of this afterhyperpolarization was increased when the number of spikes evoked by the depolarizing pulses increased.  $n$  = number of cases. Values are means  $\pm$  SEM. (D) During depolarization of the neuronal membrane potential, the rate of rise (mV/ms) of the slow depolarization increased; conversely, during hyperpolarization, it decreased. The slope of the regression line = 70.0  $\mu$ V/ms/mV ( $n$  = 14). Panels B and C illustrate the slow depolarization at two different membrane potentials (–58 mV in panel B, –49 mV in panel C). Note the same time base and calibration for panels B and C. (E) Simultaneous records of arterial pressure (AP) and intracellular recording on the same neuron as in Fig. 8 after intracellular injection of hyperpolarizing current for several minutes and intravenous infusion of sodium nitroprusside. The membrane potential was held at –67 mV. The records were triggered by the arterial pulse wave. (F) Simultaneous averages of the pulsatile AP with the neuronal membrane potential held at –71 mV during hypotension. The averages were triggered by the arterial pulse wave. Number of sweeps = 720; sample period =

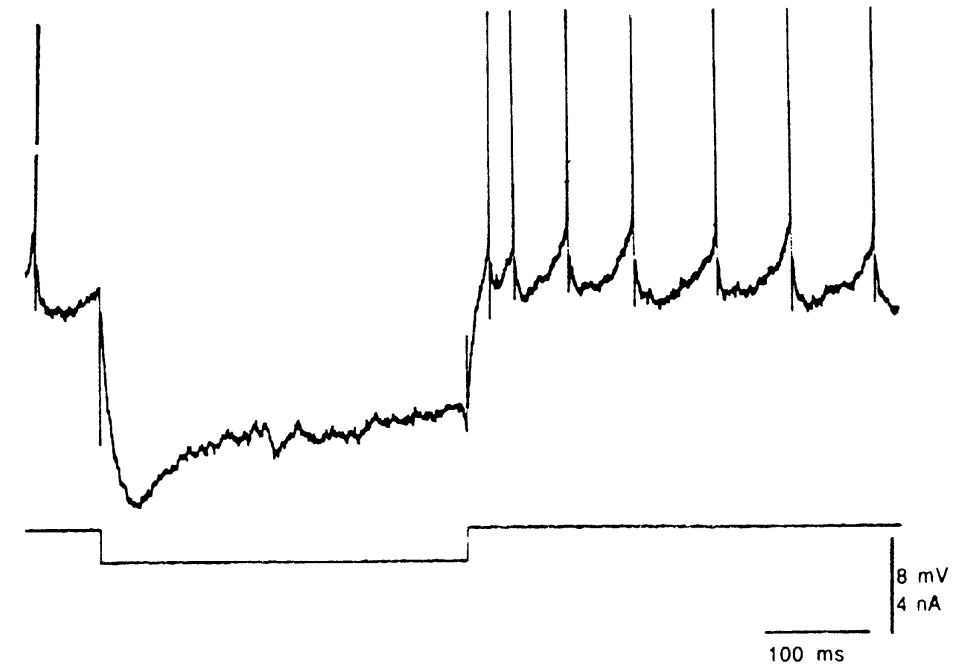
cardiac cycle at normal values of arterial pressure. However, these modulatory baroreceptor inputs are lost during hypotension. These characteristics clearly distinguish these barosensitive neurons from other RVLM neurons with barosensitive properties. Therefore, we proposed to classify these neurons as barosensitive type II (65).

The regular pacemakerlike activity observed in barosensitive type II neurons during hypotension shows more clearly that the spontaneous action potentials of these neurons are normally preceded by slow depolarizing potentials and followed by an afterhyperpolarization (Fig. 8B). The afterhyperpolarization that follows each spike is in some cases difficult to see due to the presence of the ensuing slow depolarizing potential. However, it is known that the afterhyperpolarizing potential follows single or multiple action potentials and that the amplitude of the afterhyperpolarizing potential is proportional to the number of spikes. Consequently, we found that the amplitude of the afterhyperpolarizing potential that followed depolarizing current pulses is proportional to the number of action potentials evoked by the current pulses in these barosensitive type II neurons (Fig. 9A).

We found that the slow depolarizing potential that controls the interspike activity is voltage-dependent. The corresponding slope thus increased in magnitude with depolarization of membrane potential and, conversely, decreased during hyperpolarization (Fig. 9B and C). Further, the rate of rise of the slow depolarizing potential increases when the neuron is depolarized and decreases during hyperpolarization (Fig. 9D).

The rhythmic and regular oscillations in membrane potential and the afterhyperpolarizing potential that follows each action potential during hypotension in barosensitive type II neurons are attributes of neurons with "beating," pacemakerlike activity. Further support for this view is provided by the following observations: (a) the absence of EPSP during hyperpolarization produced by passing negative current pulses up to below spike threshold level (Fig. 10); (b) interrupting the spontaneous activity by passing short pulses of hyperpolarizing current elicited a delay in the spikes occurring after the current injection in relation to the expected time based on interspike interval determined before the injection of negative current (Fig. 11A); (c) single antidromic spikes evoked on these neurons by spinal cord stimulation also interrupted the neuronal spontaneous activity, producing a delay in the spike occurring after the antidromic spike and a "resetting" of neuronal activity similar to that observed after current injection (Fig. 11B). On the other hand, when the antidromic action potential collided with a spontaneous one, no change in the firing pattern was noted (Fig. 11C).

The presence of bulbospinal RVLM neurons with pacemakerlike properties has been demonstrated in slices of rat brainstem by other laboratories. The neurons investigated by these authors showed a regular pattern of discharge in vitro. These experimenters maintained the neurons' regular firing characteristics in vitro and did not expose underlying EPSP during hyperpolarization;

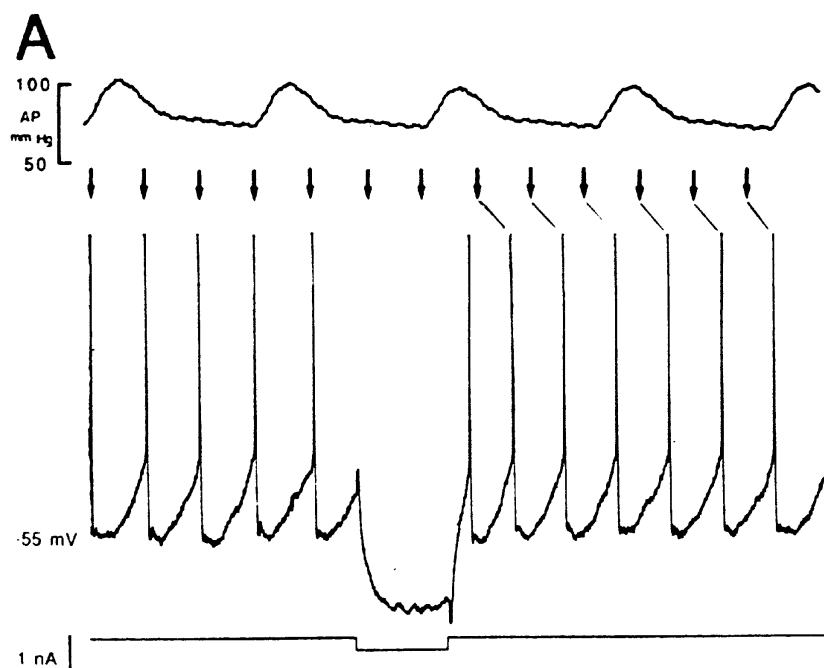


**Figure 10** Responses of a physiologically identified RVLM bulbospinal neuron to intracellular current injections. At resting membrane potential ( $-50$  mV), a hyperpolarizing current pulse produced a two-phase voltage deflection of the membrane potential. This time-dependent hang-down in the voltage deflection trace is probably due to anomalous rectification. Note that after the offset of the current pulse, the frequency of discharge was transiently increased before the neuron recovered its regular firing pattern. (From Ref. 65.)

in addition, they reset the spontaneous activity after disrupting it by hyperpolarizing current pulses (72–74). The location of these neurons appears to correspond to the nucleus reticularis rostroventrolateralis (7), the location of the neurons we characterized as barosensitive type II in our *in vivo* studies (65).

Bulbospinal neurons located in this part of the RVLM sent projections terminating only in the intermediolateral and intermediomedial columns. Therefore, it is likely that these neurons participate in the generation of tonic sympathetic nerve activity.

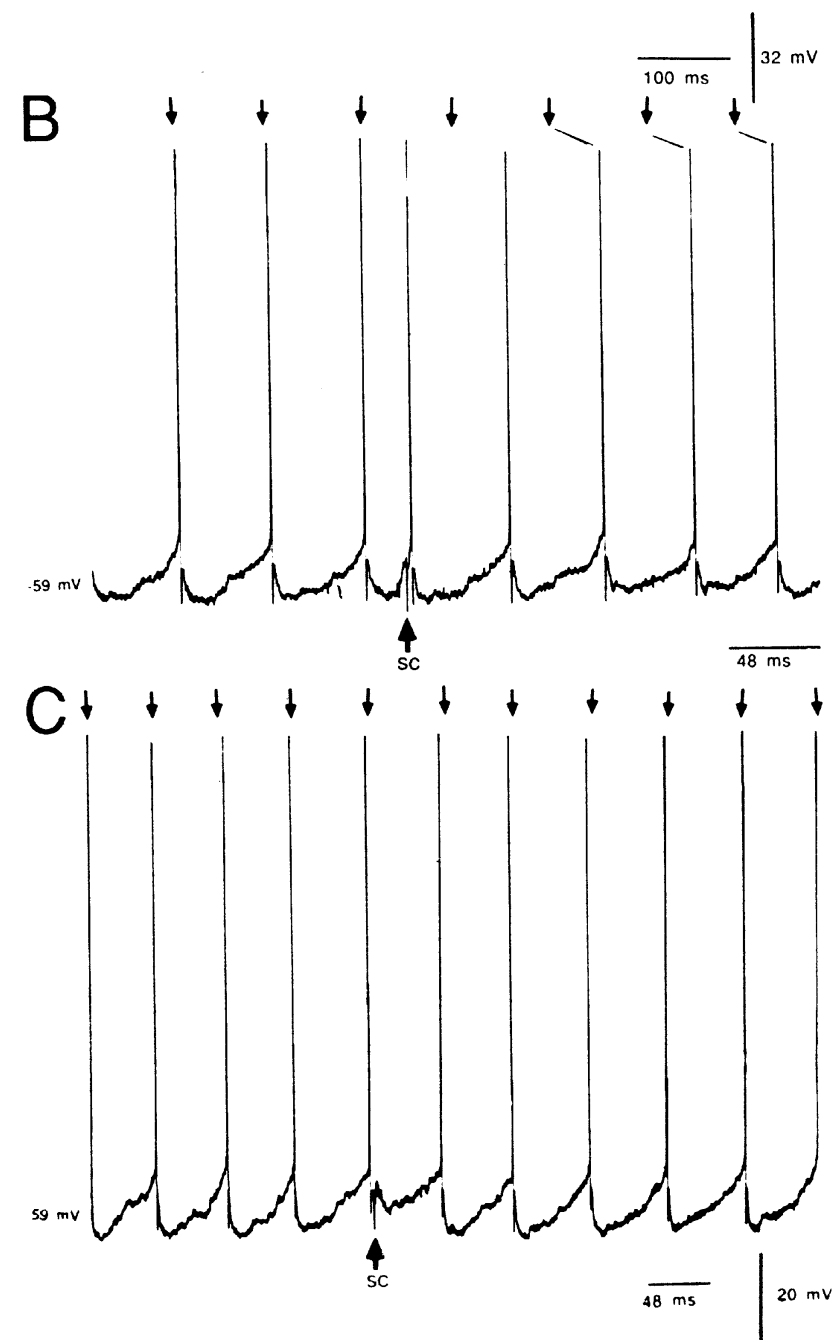
Recently, we have used a technique to record intracellular potentials from neurons in slices from the rat medulla. We identified a group of neurons in the RVLM with similar characteristics to those described for barosensitive type II neurons during hypotension. They demonstrated a very regular pattern of discharge at  $32^{\circ}\text{C}$ , the duration of action potential was in a similar range to those observed *in vivo* (1.2 to 1.7 ms), and they also demonstrated slow depolarizing potentials and afterhyperpolarizing potentials with features identical to those

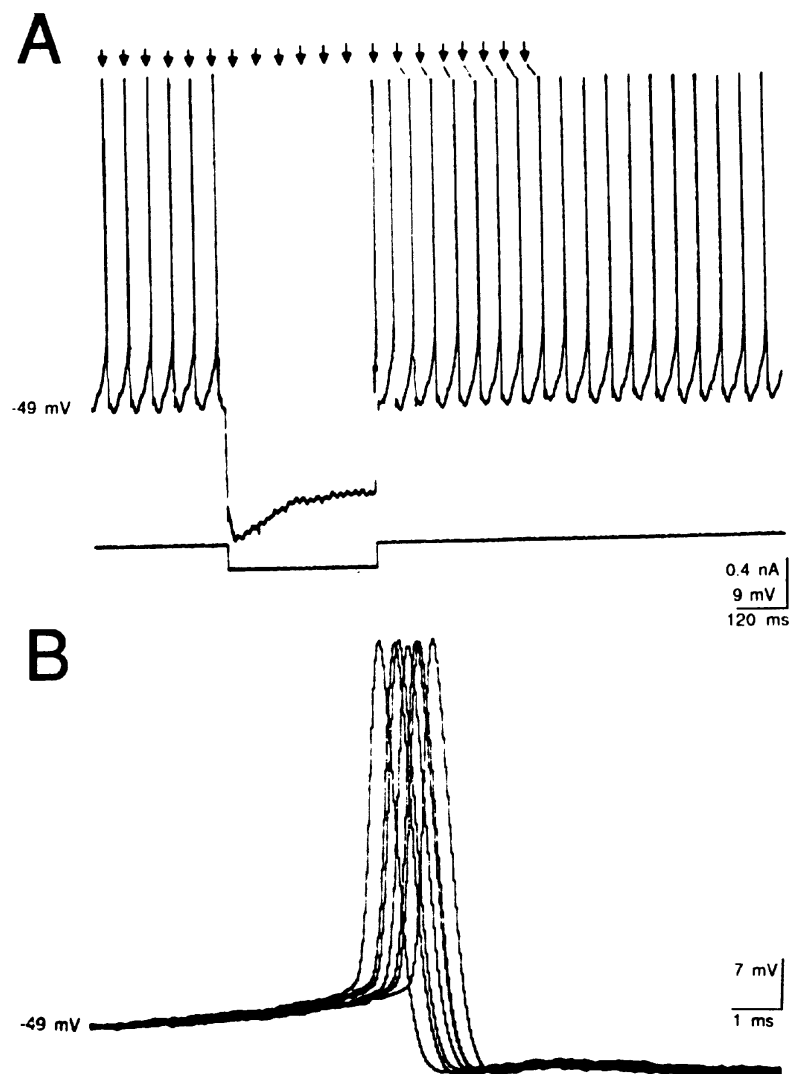


**Figure 11** Resetting of pacemakerlike activity in a barosensitive RVLM neuron. (A) Hyperpolarizing current injected in a barosensitive neuron interrupted spontaneous activity, following which the pacemakerlike activity was reset. The arrows indicate the interspike interval time after the intracellular injection. (B) Similar resetting of pacemakerlike activity after interrupting neuronal activity by an antidromically evoked spike from the thoracic spinal cord. (C) The antidromic spike collided with a spontaneous spike, and this resulted in no interruption of the rhythmic discharge. Arrows (SC) = spinal cord stimulation. (From A. R. Granata, unpublished results.)

described *in vivo*. A representative example is shown in Fig. 12. Furthermore, when the spontaneous neuronal activity was arrested by hyperpolarizing pulses, we observed a reset of neuronal activity similar to that described *in vivo* (Fig. 12A).

Another important characteristic that distinguishes these type II neurons is revealed when they are hyperpolarized by pulses of negative current. A relatively fast-developing anomalous rectification that produced a decay in the hyperpolarization was observed *in vivo* (Fig. 10). The anomaly, first described by B. Katz in potassium-depolarized muscles (75), is a conductance that increases in magnitude when the cell is hyperpolarized and decreases under depolarization. This type of conductance is present in neurons with oscillatory behavior—for example, in the substantia nigra neurons (76) or the inferior olivary neurons (77). In these neurons, the level of anomalous rectification and





**Figure 12** Intracellular recording from a regularly firing RVLM neuron from an *in vitro* slice preparation. (A) Hyperpolarizing current injected in the neuron interrupted spontaneous activity, following which the pacemakerlike activity was reset. The arrows indicate the interspike interval time after the intracellular injection. Note the absence of EPSP and the two-phase voltage deflection of the membrane potential during the hyperpolarizing period due to anomalous rectification. (B) Eight superimposed oscilloscope traces triggered by spontaneous spikes demonstrate the regularity of the firing. The action potential duration corresponded closely to that of the barosensitive type II neurons described *in vivo*. For details, see text. (From A. R. Granata, unpublished results.)

the control of the frequency of the membrane oscillation were directly related. As expected, we observed a similar type of anomalous rectification when these neurons were characterized *in vitro* (Fig. 12A). So far, we have found this type of conductance only in barosensitive type II neurons *in vivo*; hence, it has been used as an additional and important element to characterize these neurons *in vitro*.

On the other hand, in those pacemakerlike RVLM neurons investigated by Guyenet and collaborators (73,78), the authors did not report the presence of anomalous rectification when the RVLM pacemakerlike neurons were hyperpolarized, nor was it possible to detect any type of rectification in the membrane potential from the corresponding recordings (see Ref. 73, Fig. 2; or Ref. 78, Fig. 3). However, it is possible that, for various reasons, the authors of the referred papers did not hyperpolarize the neuronal membrane sufficiently to reveal the anomalous rectification. Therefore, we can only assume that the type of pacemakerlike neuron investigated by that group would not correspond with the barosensitive type II reported in our studies. Nevertheless, it is also possible to speculate that two different types of pacemakerlike neurons involved in vasomotor control may coexist in the RVLM.

In the rat, it was found that barosensitive RVLM spinal neurons with spontaneous activity locked to the cardiac cycle responded with a decrease in firing frequency when the aortic depressor nerve was electrically stimulated (24). Since the aortic nerves in the rat contain exclusively baroreceptor afferents, this is an additional and clear demonstration that these neurons are barosensitive, as concluded by the authors of that work.

We have observed that neurons characterized as barosensitive type II (according to the criteria already explained) also responded with hyperpolarization and a decrease in firing frequency during aortic nerve stimulation (A. R. Granata, unpublished results).

However, solely the demonstration that a neuron in the RVLM responds with inhibition to aortic nerve stimulation is not enough to prove its participation in the main baroreceptor reflex. The onset latency of the inhibitory response evoked by aortic nerve stimulation ranges from approximately 25 to 70 ms in the rat (24) to around 50 ms in the rabbit (56). It is therefore obvious that multisynaptic pathways are involved in mediating these inhibitory responses of RVLM spinal neurons triggered by electrical stimulation of the aortic nerve. Such polysynaptic pathways could involve either structures rostral to the pontomedullary border that do not participate in the main baroreceptor reflex arc or pathways revealed during electrical stimulation but not operative under physiological conditions. Consequently, the evoked response to aortic nerve stimulation is a complementary method of characterizing functional barosensitive sympathoexcitatory neurons but not a substitute for the modulatory synaptic inputs received by these neurons during each cardiac cycle.

