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Research report

GABA-induced responses in electrophysiologically characterized neurons within the rat rostro-ventrolateral medulla in vitro

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Abstract

Rostro-ventrolateral medulla (RVL) neurons were recorded using conventional intracellular recording techniques in brain slices maintained in vitro at 32°C and classified into 3 major groups. The first group included neurons having endogenous pacemaker-like (PL) activity with regular firing frequency (mean = 8 Hz) and a linear current–voltage relationship (I–V). The second group of neurons were slowly and irregularly firing (IF) or quiescent, presenting membrane potential oscillations and their I–V usually displayed an inward rectification. These neurons had a relatively longer action potential duration. The third group included silent neurons (S) with no apparent membrane oscillations and they differed from the first two groups by having relatively shorter action potential duration and amplitude and lower cell input resistance. When recorded with KCl-filled electrodes, the majority of silent neurons displayed a time-dependent inward rectification. With KAc-filled electrodes, irregular slow hyperpolarizing and depolarizing spontaneous potentials could be recorded primarily on PL and IF neurons, respectively. Moreover, fast spontaneous inhibitory postsynaptic potentials (PSPs) were detected in about 15% of PL and S neurons. They generally exhibited a regular pattern and were depolarizing when KCl-filled electrodes were used for recording. The amplitude of these inhibitory PSPs was reversibly reduced by the $GABA_A$ antagonists bicuculline, SR 95531 and picrotoxin. With KAc-filled electrodes, pressure-applied GABA (20 mM) evoked complex responses. In PL neurons, it consisted of a fast hyperpolarization followed by a slower depolarization that were both sensitive to SR 95531 and picrotoxin. The response was terminated by a long-lasting hyperpolarization that was reduced, but not abolished, by the $GABA_B$ antagonist CGP 35348. In IF and S neurons, GABA application usually produced a fast followed by a slow monophasic hyperpolarization and depolarization, respectively. The fast component of these responses was sensitive to the $GABA_A$ antagonists. Pressure application of isoguvacine (10 mM) always induced monophasic responses in all types of neurons recorded. Baclofen (1–30 μM) reduced the firing frequency and hyperpolarized PL and IF neurons, an effect that was antagonized by CGP 35348 (50–100 μM); however, it had little effect on silent neurons. It is concluded that RVL neurons have heterogeneous electrophysiological characteristics. Their predominant synaptic input and GABA responsiveness might be additional criteria to identify the excitatory and inhibitory elements in the RVL circuitry. All neuronal types seem to have functional $GABA_A$ and $GABA_B$ receptors; however, only a subpopulation is under tonic inhibitory control in vitro, probably from local GABAergic pacemaker interneurons. Our results further emphasize the role of GABA as an important neurotransmitter in the RVL network.

Keywords: γ-Aminobutyric acid; Rostro-ventrolateral medulla; Brainstem slice; Intracellular recording; Cardiovascular regulation; Baroreflex; Pacemaker neuron

1. Introduction

GABAergic mechanisms are involved in the central regulation of cardiovascular system (see [27] for review). In particular, reticulo- and raphe-spinal GABAergic neurons appear to contribute to the heterogeneous output from the medulla which has excitatory and inhibitory influences on sympathetic preganglionic neurons (SPNs) within the spinal cord [12]. Recently, evidence has been obtained indicating that most GABA-containing neurons with projections to the sympathetic cardioacceleratory neurons in the intermediolateral cell column of the upper thoracic cord of rats are found in the rostro-ventrolateral medulla (RVL) and the caudal ventrolateral medulla (CVL) [26]. It has also been shown that 65% of the GABAergic neurons projecting to the spinal vasomotor center originate from the RVL. Immunocytochemical studies using colchicine-treated rats have demonstrated that the RVL is heavily populated by glutamic acid decarboxylase (GAD)-labeled

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perikarya. Moreover, these studies show that a more abundant population of GAD-containing cells occupies the RVL vs the CVL [34]. This proves that in addition to the excitatory spinal projection which probably constitutes the greatest proportion of bulbospinal neurons [22], the RVL contains GABAergic neurons which act as local inhibitory neurons, or project spinally to inhibit SPNs. These intrinsic GABAergic neurons might provide tonic inhibition of RVL vasomotor neurons or mediate vasodepressor responses from baroreceptors via a direct input from the nucleus tractus solitarius (NTS) since there is no evidence for a direct GABAergic afferent projection from the NTS to the RVL [24].

Electrophysiological evidence has accumulated that RVL neurons are under tonic GABAergic inhibition. Microinjection of $GABA_A$ and $GABA_B$ agonists in the RVL inhibits the neurons and causes a depressor response and a decrease in heart rate while $GABA_A$ and $GABA_B$ antagonists produce the opposite effects [2,33,38,48].

Defects in the GABAergic system might contribute to the exaggerated sympathetic tone, and in turn, to the circulatory changes involved in the initiation, expression, or maintenance of neurogenic hypertension. Centrally acting antihypertensive drugs like clonidine [7] and propanolol [31], have been shown to stimulate the GABAergic system activity in spontaneously hypertensive rats. Deficiency in the GABAergic input into the RVL originating from the CVL has been suggested to contribute to the elevation of arterial pressure in the SHR [38].

It is well documented that the RVL contains chemically and physiologically different population of neurons. Therefore, our study was designed to further characterize the different groups of neurons in this region and investigate the responses to exogenously applied and synaptically released GABA in each group of these neurons using intracellular recording in vitro.

Our data show that a population of RVL neurons receives regular GABAergic inhibitory postsynaptic potentials (IPSPs) probably resulting from the spontaneous activity of local GABAergic pacemaker interneurons. In addition, we are the first to characterize, using intracellular recordings, a group of slowly and irregularly firing neurons having relatively larger spike duration. We have also shown that the different groups of RVL neurons exhibit different GABA responsiveness. This electrophysiological and pharmacological distinction is probably of considerable significance in order to predict the chemical identity of RVL neurons and their physiological function.

A preliminary report of part of this study has been previously published in abstract form [9].

2. Materials and methods

Wistar rats (50–100 g) were anesthetized with ether and decapitated. The brainstem along with the cerebellum were quickly removed and placed for 30 s in cold (2–4°C) modified artificial cerebrospinal fluid (mACSF, see below) in which sucrose 248 mM was substituted for NaCl 124 mM and equilibrated with 95% O_2/5% CO_2. The tissue was trimmed with a razor blade and a block containing the medulla and a part of the cerebellum was glued with cyanoacrylate in front of an agar block on a Petri dish and covered with the mACSF. Two to 3 transverse slices (400 μm thick) containing the RVL were sectioned using an Oxford Vibratome and left to recover in mACSF at room temperature for 30 min. A slice was then transferred to the recording chamber and perfused at a rate of 1.5–2 ml/min with oxygenated ACSF (pH = 7.35) of the following composition (mM): NaCl 124, KCl 2, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, KH_2PO_4 1.25, and D-glucose 10. The temperature in the recording chamber was raised slowly from room temperature up to 31–32°C and the slice was allowed to equilibrate for 1 h before commencement of recording. Signals were recorded using a high-impedance bridge amplifier (Axoclamp-2B, Axon Instruments Inc.) filtered at 10 KHz bandwidth and displayed on an oscilloscope (Tektronix 5116). The output signal was also directed to a graphic thermal recorder (Gould TA240) and a digital tape recorder (DTR-1201, Biologic) for storage and later analysis. Data analysis was performed using a personal computer equipped with Digidata 1200 analog/digital interface and pClamp software (Axon Inst., Foster City, CA). Electrodes were filled with KAc (2 M) (80–150 MΩ) or in some experiments with KCl (3 M) (35–70 MΩ) and pH was adjusted to 7.4 with HAc and KOH, respectively. Electrodes were guided towards the RVL with the assistance of a dissecting microscope and were advanced using a micromanipulator (Narishige, Japan). The approximate position of the neurons impaled within the slice was estimated from the scaling of the micromanipulator.

Drugs were dissolved in ACSF and applied via a 3-way tap system, by changing the superfusion solution to one which only differed in drug content. The delay between turning the tap and the first arrival at the tissue of the exchanged solution was about 20 s. The ratio of flow rate to bath volume ensured complete exchange within 1 min. In order to avoid the problem of desensitization, GABA, isoguvacine, and glutamate (10–20 mM) were applied by pressure using a multichannel picospritzer (General Valve Corporation). Pressure applications had a duration of 200 ms to 2 s and a pressure of 10–20 d.p.i. Pressure pipettes were made by breaking the tip of intracellular sharp electrodes to about 3 μm diameter.

The following compounds purchased from Sigma Co. were used: GABA, isoguvacine, (−)-baclofen, L-glutamate, bicuculline methiodide, picrotoxin, strychnine, nipecotic acid. CGP 35348 and SR 95531 were kindly offered by Ciba-Geigy and Sanofi Recherche, respectively.

The input resistance of RVL neurons was determined by measuring the asymptotic voltage drop caused by small

hyperpolarizing currents. Spike amplitude and duration were calculated from the threshold to the peak and to the corresponding repolarization phase of the action potential, respectively. In silent neurons, these parameters were evaluated using the minimum current pulse (20 ms) needed to evoke an action potential. The resting membrane potentials were calculated upon withdrawal from cells. Changes in membrane potential due to drug application were offset by direct current injection to show effect on membrane input resistance. Statistical comparisons were made with two-tailed Student's *t*-test using Origin 3.0 program. Data are expressed as mean ± S.D.

3. Results

3.1. Electrophysiological characteristics

Stable intracellular recordings were obtained from 190 neurons in the RVL identified by their location ventromedial to the compact rostral portion of the nucleus ambiguus visible in slices by transillumination. The area investigated in this study was the same as defined by Li and Guyenet [19,20]. Neurons of the RVL could be divided into 3 major groups: pacemaker-like (PL), irregular firing (IF) and silent neurons (S).

3.1.1. Pacemaker-like neurons

Neurons were considered to have pacemaker-like activity if they fulfilled one of the following criteria: (1) they had endogenous pacemaker activity that did not result from excitatory postsynaptic activity and which could be reset with intracellular injection of current; or (2) they demonstrated at rest spontaneous, relatively regular membrane potential oscillations which were mostly subthreshold for the generation of action potentials. When minimum positive current was injected into these neurons in order to reach action potential threshold, regular firing (7–9 Hz) was obtained. Moreover, the same effect was obtained in a reversible manner when switching the extracellular concentration of K^+ ions from 3.25 to 6 mM.

The firing frequency of PL neurons ranged from 4–15 Hz at 31°C (8.5 ± 2.8 Hz, $n = 33$), and their membrane potential depolarized from −62 to −53 mV, the apparent threshold for spike initiation. Their spike amplitude and duration were 58.2 ± 6.1 mV and 0.99 ± 0.27 ms ($n = 46$), respectively (Fig. $1A_1$, A_2). They displayed a linear current–voltage relationship with an average membrane input resistance of 114 ± 33 MΩ ($n = 22$) (Fig. $1A_3$, A_4). The membrane time constant was 16.2 ± 5.8 ms ($n = 22$).

3.1.2. Irregular firing neurons

IF neurons ($n = 44$) had slower and irregular discharge pattern and in some cases, they were quiescent showing only membrane potential oscillations that did not reach the threshold for spike generation; thus, their firing frequency

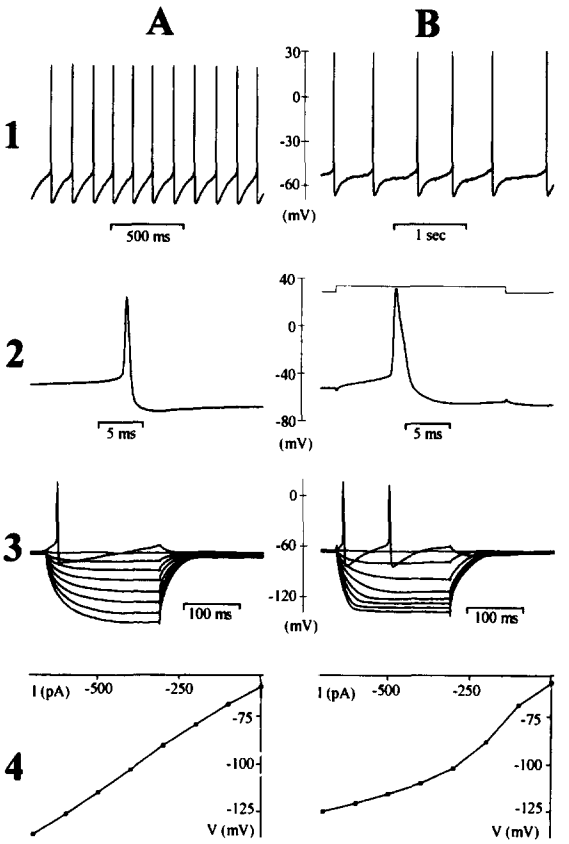

Fig. 1. Electrophysiological properties of pacemaker-like (PL) (A) and irregular firing (IF) (B) neurons. A_1 and B_1 represent the mode of discharge of the two types of neurons. Note the regular pattern in PL neurons and the relatively slow and irregular discharge of IF neurons. In A_2 and B_2, the spike shape is illustrated. Note the relatively longer spike duration of the IF neuron and the shoulder present on the repolarizing phase of the action potential. A_3, A_4 and B_3, B_4 illustrate the voltage responses to steps of hyperpolarizing current pulses (−0.7 to 0.1 nA, steps of 0.1 nA) and the corresponding $I-V$ relationship in the two types of neurons, respectively. Note that the PL neuron has a linear $I-V$ relationship whereas the IF neuron demonstrates an inward rectification at hyperpolarized potentials. KAc-filled electrodes were used for all the recordings in this figure.

ranged from 0 to 8 Hz. Unlike PL neurons, quiescent neurons of this group usually exhibited irregular firing when their membrane was depolarized by current injection (Fig. $1B_1$) These neurons were mostly distinguished by their action potential duration which was significantly different from the other two neuronal populations ($P < 0.01$), and ranged from 1.3 to 2.8 ms (1.74 ± 0.43 ms, $n = 44$). However, some overlap was sometimes observed; therefore, the firing pattern and other membrane properties were used to classify the neurons. Their action potential amplitude was 60.5 ± 7.1 mV ($n = 44$) and their resting membrane potential was −58.7 ± 4.9 mV ($n = 18$). The neurons of this type sometimes presented a shoulder on the repolarization phase of their action potential (Fig. $1B_2$). This was more obvious when the same neurons were recorded at room temperature (22–24°C). Their membrane input resistance and time constant were 139 ± 29 MΩ and

13.1 ± 4.6 ms ($n = 18$), respectively. The majority of the IF neurons presented an inward rectification evident from their voltage–current relationship (Fig. $1B_3$, B_4).

3.1.3. Silent neurons

In addition to being quiescent, these neurons ($n = 100$) were mostly distinguished from pacemaker neurons by having action potentials of smaller amplitude (49.6 ± 7.9 mV) and duration (0.76 ± 0.17 ms) ($n = 81$) with no apparent membrane oscillations, in addition to a smaller input resistance (64 ± 34 MΩ) and membrane time constant (10.4 ± 5.8 ms) (Fig. $2A_1$, A_2). These parameters were significantly different from those of PL and IF neurons ($P < 0.01$). The majority of these neurons (65%) had low membrane input resistance (20–65 MΩ) and a rather negative resting membrane potential (−68 ± 6 mV, $n = 47$).

When recordings were made using KCl-filled electrodes, the majority of silent neurons (more than 75%) demonstrated a significant time-dependent anomalous rectification, which was almost never encountered with KAc-filled electrodes (Fig. 2C).

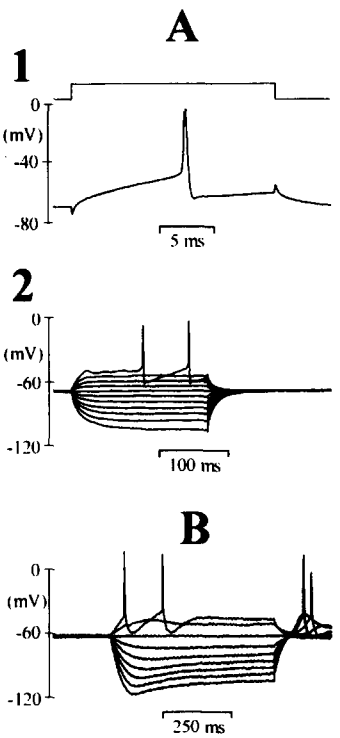

Fig. 2. Electrophysiological properties of a silent neuron (S). A_1 illustrates an action potential evoked by a depolarizing current pulse (0.4 nA) in a silent neuron recorded with a KAc-filled electrode. Note the relatively small amplitude of the action potential (43 mV) and short duration (0.65 ms). In A_2, the membrane response to different intensities of current injection (−0.6 to 0.3 steps of 0.1 nA) is illustrated. Note the low membrane input resistance (60 MΩ). B: same as in A_2, in a silent neuron recorded with a KCl-filled electrode. Note that the neuron developed a time-dependent anomalous inward rectifier at hyperpolarizing levels.

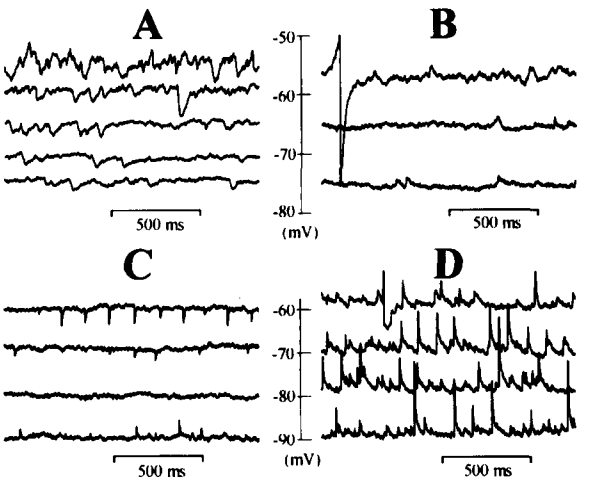

Fig. 3. Spontaneous synaptic activity in RVL neurons. A: relatively slow hyperpolarizing postsynaptic potentials recorded in a PL neuron (KAc-filled electrode). These events occurred randomly and their amplitude decreased with hyperpolarization; however, the frequency of their occurrence did not seem to be affected. B: depolarizing events in an IF neuron at different membrane holding potentials (KAc-filled electrode). C and D: recordings in two different silent neurons recorded with KAc and KCl-filled electrode, respectively. In C, the neuron had a resting membrane potential of −68 mV. The amplitude of the IPSPs increased with membrane depolarization and reversed in polarity at approximately −78 mV. In D, the neuron had a resting membrane potential of −70 mV. When the membrane potential was depolarized, the PSPs sometimes triggered an action potential. The amplitude of these depolarizing PSPs increased with membrane hyperpolarization.

3.2. Spontaneous postsynaptic activity

In all types of RVL neurons described above, we recorded spontaneous postsynaptic potentials (PSPs). Irregular relatively slow hyperpolarizing potentials (time to peak ≅ 30 ms) were recorded in most PL neurons when they were silenced by negative current injection. These potentials were mixed with membrane oscillations near threshold; however, further hyperpolarization of these neurons did not affect their frequency, but reduced their amplitude (Fig. 3A); therefore, they probably reflected synaptic activity. This activity was neither sensitive to the $GABA_A$ antagonist SR 95531 (100 μM, $n = 2$), bicuculline (30 μM, $n = 3$) nor to the $GABA_B$ antagonist CGP 35348 (300 μM, $n = 2$) and was observed in PL neurons recorded with either KCl or KAc-filled electrodes. The pharmacological identity of this activity was not further pursued.

On the other hand, IF neurons received primarily depolarizing events (KAc-filled electrodes). Similarly to PL neurons, these events became more evident when their membrane was hyperpolarized by negative current injection (Fig. 3B). However, since only a small percentage of these neurons exhibited such activity, the effect of TTX was not investigated.

When recordings were made using KAc-filled electrodes fast hyperpolarizing PSPs were recorded in about

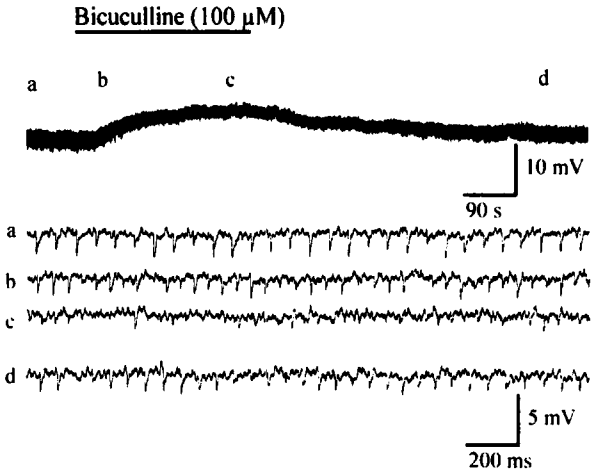

Fig. 4. Effect of bicuculline on spontaneous regularly occurring IPSPs in a silent RVL neuron (KAc-filled electrode). Top: bicuculline induced a depolarization of the membrane potential and a decrease in the amplitude of inhibitory postsynaptic activity. Bottom: the lower 4 traces represent the same activity at an expanded time scale in control (a), at the beginning (b) and during bicuculline application (c) and during washout (d). Note the increase in frequency of IPSPs in b and the decrease of their amplitude in c.

15% of the PL and S neurons. They reversed in polarity at a membrane potential varying between −65 and −80 mV (Fig. 3C). Most of the PSPs were abolished in the presence of TTX (1 μM) (PL, $n = 2$; S, $n = 6$). Some PSPs of small amplitude remained, suggesting that they are miniature events. An interesting observation was made when quiescent cells having low membrane input resistance were recorded at temperatures varying from 31 to 22°C. When the temperature was decreased in the chamber, cell input resistance increased and in 4 out of 7 neurons, IPSPs appeared with higher amplitude at more regular intervals and with a frequency that decreased with temperature. This was associated with an increase in spike duration and amplitude (not shown).

With KCl-filled electrodes, depolarizing PSPs were observed more frequently (about 50%) in PL and S neurons. They often appeared to occur at fairly regular intervals and sometimes together with some irregular miniature events (Fig. 3D).

SR 95531 (20–50 μM) ($n = 3$), bicuculline (30–120 μM) ($n = 4$) and picrotoxin (100 μM) ($n = 4$) inhibited or reduced to a large extent the amplitude of the spontaneous IPSPs. In neurons where regular IPSPs were recorded, these $GABA_A$ antagonists increased at first the frequency of the IPSPs before any antagonism took place and produced a membrane potential depolarization (Fig. 4). In all types of neurons, the $GABA_A$ antagonists depolarized the cells with sometimes an increase in membrane input resistance. This was observed when recordings were made using either KCl or KAc-filled electrodes. Pressure application of glutamate (10 mM) in the vicinity of the recorded neurons of all types depolarized their membrane potential (up to 30 mV) and revealed inhibitory and excitatory PSPs ($n = 6$) and increased the frequency of baseline IPSPs ($n = 2$) (not shown). Pressure-applied GABA reduced the frequency of regularly occurring IPSPs or shunted them ($n = 4$) (not shown). Strychnine (10 μM) inhibited part of the IPSPs in 2 of the 5 neurons tested while no significant effect was observed in the remainder.

3.3. $GABA_A$ responses

GABA applied by pressure evoked responses only in neurons which were located within the superficial layer of the slice (150 μm). We preferred the pressure application in order to avoid problems of desensitization caused by bath application of GABA which in concentration up to 1 mM produced little change on membrane potential in silent neurons ($n = 5$) and only a slight decrease in the discharge frequency of regular or irregular firing neurons ($n = 4$) (not shown).

Pressure application of GABA (20 mM) produced a fast depolarizing response (up to 20 mV) in all neurons recorded with KCl-filled electrodes ($n = 15$). This was followed in some cases by a hyperpolarization which was mostly significant in PL and IF neurons.

In neurons recorded with KAc-filled electrodes, GABA produced complex responses consisting of fast and slow components. The nature of the GABA responses depended on the location of the neuron within the slice, the dose of GABA delivered and the type of neuron recorded (Fig. 5) as given below.

In PL and IF neurons recorded with KAc-filled elec-

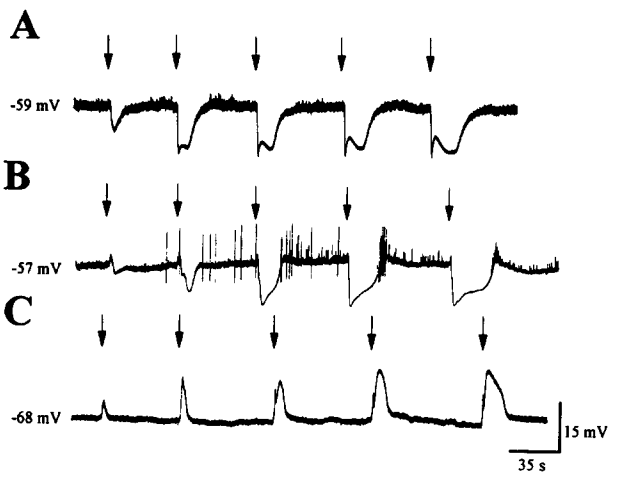

Fig. 5. Dose-dependent responses in PL (A), IF (B) and S (C) neurons to GABA applied by pressure. The duration of the application (indicated by arrows) was doubled each time starting from 50 ms. Note that in the PL neuron (A), increasing the amount of GABA delivered revealed a triphasic response while such a response was not observed in the IF neuron (B). On the other hand, GABA induced a depolarization in the silent neuron (C). All neurons were recorded with KAc-filled electrodes and GABA responses were recorded at the resting membrane potential except in A, where the PL neuron was silenced by a small negative-current injection.

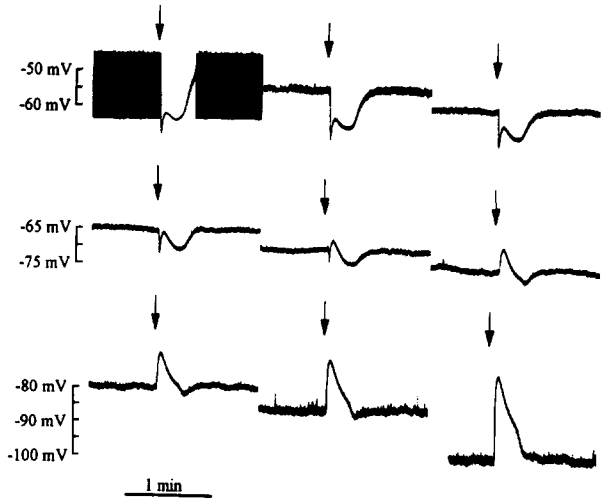

Fig. 6. Reversal potential of GABA multiphasic responses in a PL neuron (KAc-filled electrode). GABA application (500 ms, arrows) at different holding potentials in the bridge mode. The triphasic response to GABA consisted of a fast hyperpolarization and a long-lasting one which reversed around −75 and −90 mV. A depolarizing phase was embedded between these two hyperpolarizing components. Its amplitude (relative to the fast hyperpolarization) seemed not to be affected by membrane hyperpolarization. A clear separation of the first two phases was evident at a holding potential of −70 mV, where both the fast hyperpolarization and the depolarization could be observed.

trodes, isoguvacine (10 mM) or GABA induced monophasic responses that reversed in polarity at -73.5 ± 0.9 mV ($n = 4$). In all cases, this was associated with a marked decrease in membrane input resistance. When higher doses of GABA were delivered by increasing the duration of pressure application, complex multiphasic responses were obtained especially in PL and S neurons. Unlike GABA, isoguvacine always produced a monophasic response even when increasing the dose applied ($n = 8$).

In PL neurons, a sufficient dose of GABA tended to evoke multiphasic responses ($n = 20$). These responses consisted of a fast hyperpolarization, a slower depolarization and a GABA dose-dependent long-lasting hyperpolarization. When the membrane holding potential was manipulated by negative constant-current injection, the fast and long-lasting hyperpolarizing components reversed in polarity at -75.0 ± 0.4 mV and -87 ± 1.5 mV ($n = 5$), respectively. However, the intermediate depolarizing shoulder could not be reversed by membrane hyperpolarization as if this phase was far from being clamped by current injection (Fig. 6). In IF neurons, there were no depolarizing phase whatever the dose of GABA applied and the response usually consisted of a fast hyperpolarization and a slower long-lasting one (Fig. 5B).

Complex responses to pressure-applied GABA were sometimes obtained with silent neurons and they consisted of intermixed hyperpolarization and depolarization phases ($n = 4$). However, in most of the silent neurons having a rather negative potential, GABA usually produced a monophasic depolarizing response ($n = 6$) (Fig. 5C).

Bath application of picrotoxin (100 μM) or SR 95531 (20–60 μM) affected the fast components of GABA responses in PL and IF neurons (Fig. 7A,B). They com-

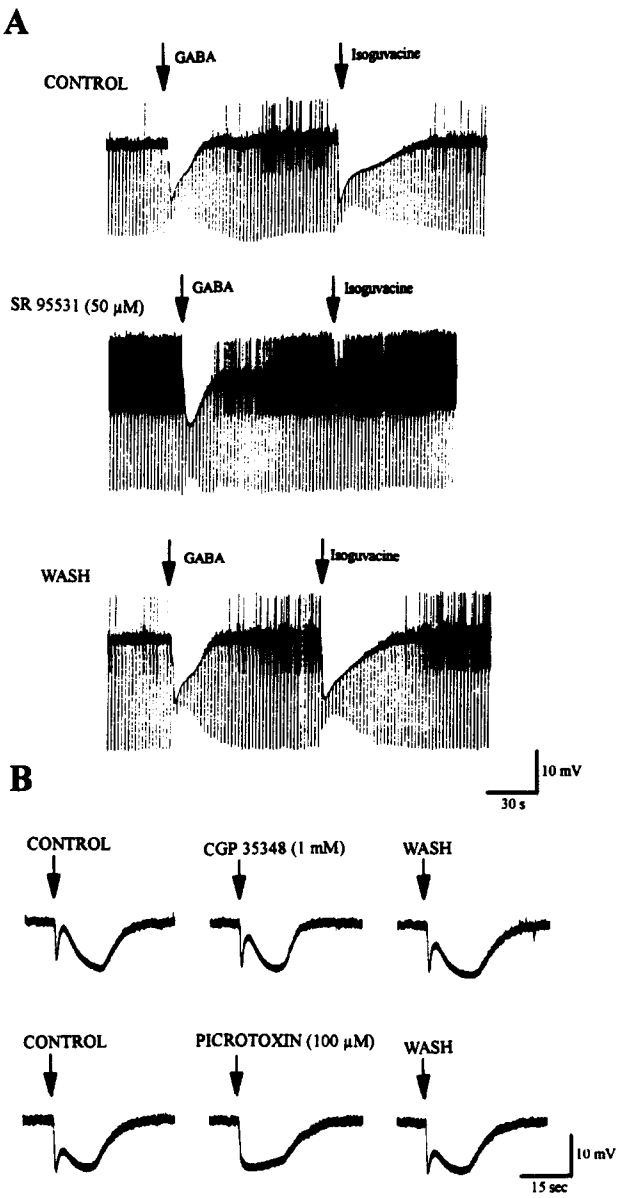

Fig. 7. Pharmacological properties of GABAergic responses in RVL neurons (KAc-filled electrode). A: effects of GABA and isoguvacine in an IF neuron and antagonism by SR 95531. Isoguvacine produced a longer response that was abolished almost completely by the specific $GABA_A$ antagonist, whereas an SR 95531-insensitive hyperpolarization response persisted after GABA application probably indicating a residual activation of $GABA_B$ receptors. Note the marked decrease in R_{in} after all GABA agonists applications, and the small depolarization and increase in firing frequency during SR 95531 application. Recovery in A and B was obtained after 20 min washout. B: multiphasic GABA response obtained in a PL neuron silenced by small current injection to a holding potential of −62 mV. The $GABA_B$ antagonist CGP 35348 slightly reduced the long-lasting hyperpolarizing component of the GABA response, whereas the $GABA_A$ antagonist SR 95531 reduced the fast hyperpolarizing phase and suppressed the depolarizing one. The GABA agonist pressure application is indicated by arrows.

pletely abolished the isoguvacine responses in both PL ($n = 2$) and IF neurons ($n = 2$) (Fig. 7A) and significantly reduced the depolarizing component of the GABA responses in PL neurons ($n = 4$) (Fig. 7B) and S neurons ($n = 2$). Picrotoxin (100 μM) was the most effective and it almost completely washed out in 20 min. Application of CGP 35348 (0.5–1 mM) reduced only slightly the long-lasting hyperpolarizing component in PL ($n = 3$) (Fig. 7B) and IF ($n = 2$) neurons. Bath application of nipecotic acid (1–5 mM) prolonged all the components of GABA responses, in particular the long-lasting hyperpolarizing component ($n = 3$). This effect was mimicked by increasing the duration of GABA application (not shown).

3.4. $GABA_B$ responses

Baclofen (3 μM) hyperpolarized the membrane potential of pacemaker neurons by 5 ± 2 mV ($n = 10$) when tested from resting potential. This effect was dose-dependent (range 1–30 μM) (not shown). Low concentrations of baclofen (1 μM) decreased the firing frequency of all pacemaker neurons tested or inhibited their spontaneous activity, therefore allowing to better distinguish IPSPs or EPSPs distinct from membrane oscillations on PL and IF neurons, respectively (Fig. $8A_1$, B). The baclofen response was associated with a decrease in membrane input resistance (25% decrease, $n = 4$) (Fig. $8A_3$). Baclofen (1 μM) response was antagonized about 70% by CGP 35348 (50 μM) ($n = 3$) (Fig. $8A_2$). When CGP 35348 was administered alone, it provoked a small increase in frequency in 3 out of 3 PL and 2 out of 2 IF neurons and this effect was reversible. Baclofen (up to 50 μM) slightly hyperpolarized the silent neurons when tested from resting potential and inhibited their postsynaptic activity with small changes in membrane potential (Fig. 8C, D_1). When positive current was injected into these neurons in order to induce them to fire action potentials, an inhibition of the evoked discharge was observed after baclofen application ($n = 4$). However, even in these conditions, the hyperpolarization induced by baclofen remained relatively small (Fig. $8D_2$).

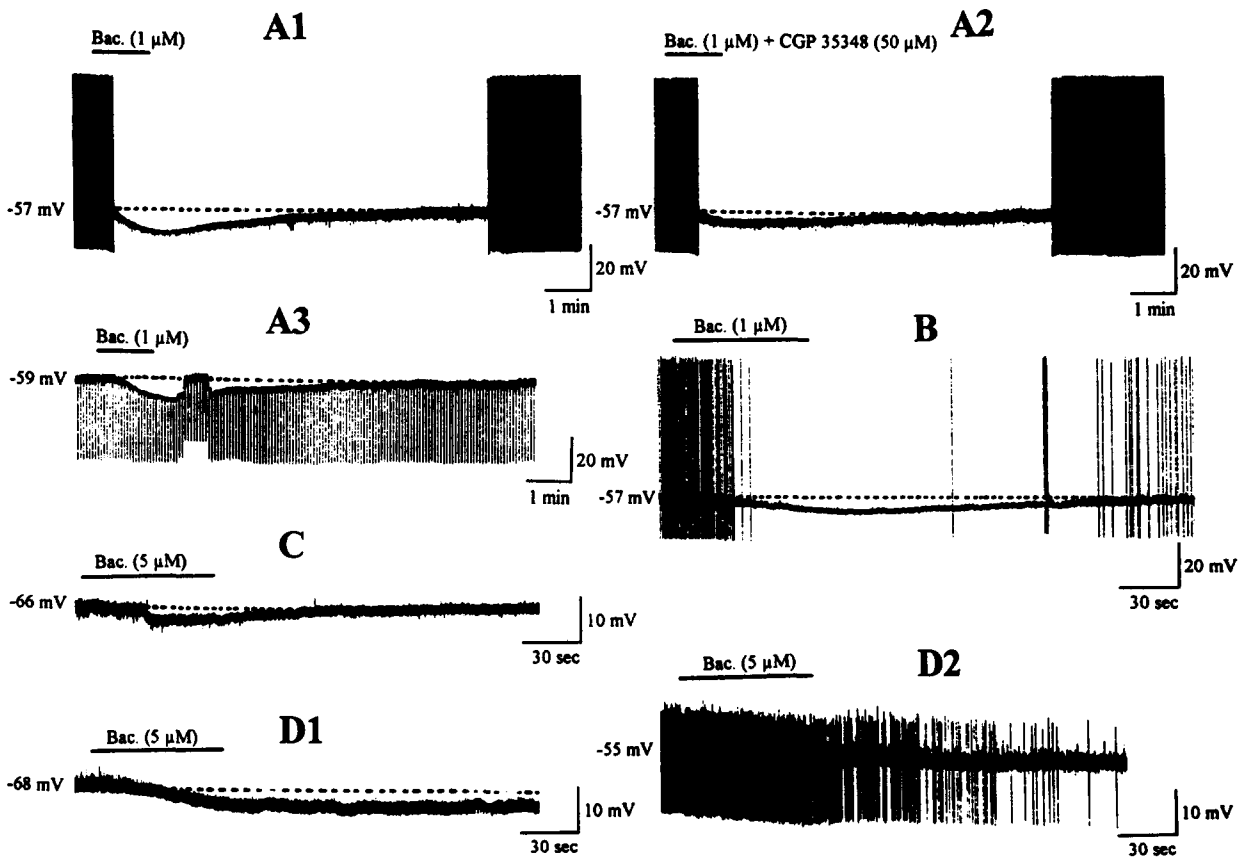

Fig. 8. Effects of baclofen on RVL neurons (KAc-filled electrode). A: baclofen response in a PL neuron. A_1 is control, A_2 during application of CGP 35348. In A_3, the neuron was silenced by negative current injection. The effect of baclofen on membrane input resistance was monitored by injecting negative-current pulses (duration 50 ms, amplitude −0.2 nA, 0.5 Hz). The membrane potential was manually clamped during the response to control level to show the decrease in cell input resistance. B: baclofen response on an IF neuron. C and D_1: baclofen response on two silent neurons whose membrane potential demonstrated negative and positive PSPs, respectively. D_2: baclofen was applied in the same cell as D_1 after depolarizing the membrane potential to threshold level for generation of action potential.

4. Discussion

Our results constitute the first detailed electrophysiological classification of the different types of neurons that can be encountered in the RVL, based on a large sampling of neurons. Moreover, they demonstrate that a GABAergic system is involved in the regulation of the activity of RVL neurons, namely by demonstrating the presence of functional $GABA_A$ and $GABA_B$ receptors on the membranes of both pacemaker and silent cells in this brain region. In addition, they show for the first time that some of these neurons receive a tonic GABAergic synaptic activity which probably results from local GABAergic pacemaker neurons.

4.1. Electrophysiological characteristics

This study confirms the existence of RVL neurons with intrinsic pacemaker activity as has been reported by other investigators [13,16,45]. It has been reported [43] that these neurons do not show regular EPSPs when hyperpolarized and low Ca^{++}, high Mg^{++} solutions do not affect their discharge pattern. It is important to mention that in our study we have observed that certain non-pacemaker neurons exhibiting membrane potential oscillations assumed a regular firing pattern by small positive current injection without any accommodation and with a frequency similar to that of PL neurons. A similar observation has been made by Kangrga and Loewy [13]. It is possible that these 'silent' neurons might be true pacemaker but for unknown reason, are just under the edge of the spiking activity. Beside the mode of discharge of these neurons, in our classification we have taken into account the action potential duration and amplitude as well as the current–voltage relationship.

To our knowledge, this is the first intracellular study that clearly identifies a population of RVL neurons having a slow and irregular firing pattern or quiescent activity presenting membrane oscillations. Moreover, these neurons, which constitute about 25% of the total neurons recorded in this study, had a relatively long duration action potential. It is interesting to notice that in many other monoaminergic areas of the brain, like the ventral tegmental area [11], substantia nigra zona compacta [15], locus ceruleus [49] and the nucleus raphe magnus [29], monoaminergic neurons have been reported to have a large spike width as compared to other neurons in the same areas. On the other hand, it has been reported that C1 adrenergic clonidine-sensitive neurons have a relatively slower discharge rate in vivo [1,40]. It might, therefore, be possible that the neurons classified as IF in our study belong to the catecholaminergic cell group of the RVL.

4.2. GABA responses

An interesting observation in this study was that pressure-applied GABA on RVL neurons evoked complex responses. The amount of GABA delivered and the position of the neuron recorded within the slice were some of the factors which appeared to affect the nature of the GABA responses. Often, GABA applied to the surface of the slice near the recording electrode was unable to evoke any response. It is therefore possible that GABA did not reach their membrane surface or was degraded or reuptaken by glia. In addition, GABA was applied in the absence of synaptic blockers: it is therefore possible that some complications were added by the release or the inhibition of release of neurotransmitters.

Typical multiphasic GABA responses were obtained on PL neurons. They usually consisted of a fast hyperpolarization followed by a slower depolarization and they were sensitive to $GABA_A$ antagonists. A slower long-lasting CGP 35348-sensitive hyperpolarization terminated the response, suggesting the involvement of $GABA_B$ receptors. Similar actions of GABA have been described in other brain preparations, like the visual cortex [35], hippocampal pyramidal neurons [3], and intradendritically recorded Purkinje cells [36]. The biphasic $GABA_A$ responses have generally been attributed to a differential somato-dendritic chloride equilibrium potential which was more positive in the dendritic tree with respect to the soma. This point remains to be clarified in our preparation in future studies.

The final concentrations of the pressure-applied GABA agonists were unknown in our conditions. This could explain the incomplete blockade of GABA responses by the bath applied $GABA_A$ and $GABA_B$ antagonists at saturating doses of GABA. Even though CGP 35348 had only a slight effect on the long-lasting hyperpolarizing component of GABA response, we still suspect that this component was due to an activation of $GABA_B$ receptors because this phase reversed in polarity at -87 mV near the equilibrium potential of K^+ ions. Moreover, it was only produced at saturating doses of GABA and it was never obtained with isoguvacine, the specific $GABA_A$ agonist.

On the other hand, $GABA_B$ receptor activation by baclofen induced an effect both on resting membrane potential and on the synaptic activity in RVL neurons. More prominent direct postsynaptic effects of baclofen were observed on PL and IF neurons and were associated with a decrease in cell input resistance suggesting that the hyperpolarizing responses might be mediated via opening of a K^+ conductance as has been reported for NTS neurons [6].

Baclofen inhibited presynaptic activity on silent neurons with little postsynaptic effect. This might result from their negative membrane potential associated to a low input resistance. Indeed, baclofen slowed the spiking activity of these neurons when depolarized by current injection; therefore, the accommodation of the evoked firing induced by baclofen suggests that these neurons might have a functional $GABA_B$ receptor. The presence of $GABA_B$ receptors in the RVL has recently been reported [20]. Our study

shows, moreover, that in the RVL, functional $GABA_B$ receptors are present predominantly on neurons having endogenous spontaneous activity, i.e. PL and IF neurons.

4.3. GABAergic spontaneous synaptic activity

Immunocytochemical labeling studies have shown that GABAergic terminals innervate adrenergic as well as non-adrenergic cell bodies, dendrites, axons, and axon terminals within the RVL [25]. All types of neurons recorded in our study seem to receive a GABAergic synaptic activity. Granata and Kitai [8] have recorded intracellularly in vivo a group of bulbospinal RVL neurons characterized by chloride-dependent IPSPs locked to the cardiac cycle which disappeared during baroreceptor inactivation. The present observation that spontaneous IPSPs occurred at regular intervals in RVL neurons is of importance because these synaptic events probably reflect the regular discharge of presynaptic inhibitory interneurons. These presumed GABAergic neurons might be interneurons acting just locally within the RVL or they might be also bulbospinal innervating the SPNs. In all cases, it is reasonable to assume that they might play a role in the baroreflex arc by inhibiting the sympathoexcitatory drive. However, it is also very probable that GABA in the RVL plays an important role in other physiological functions such as respiratory control. Therefore, we will use the terminology 'interneuron' to designate them, even though no labeling was performed to show their exact nature.

The IPSPs were not completely abolished by bicuculline up to 120 μM. A similar observation has been made in the locus ceruleus [28] where bicuculline methiodide (80–100 μM) reduced, but did not abolish, the effects of 600 μM of GABA or higher. Therefore, the concentration of GABA released in the synaptic cleft might be high enough to compete with bicuculline at the concentration used. On the other hand, glycine could be responsible for the remaining IPSPs since strychnine abolished part of the IPSPs in the few neurons tested. This is in agreement with the data reported previously [2,33] which suggest that glycine is unlikely involved in mediation of the tonic inhibition since the glycine antagonist strychnine injected in the RVL did not affect basal blood pressure.

Cell input resistance seems to influence the size and consequently the detection of IPSPs. Our observation that decreasing the temperature from 32 to 24°C revealed previously undetected synaptic activity is in large part a consequence of the increase in cell input resistance. The broadening of the spike of presynaptic pacemaker interneurons at lower temperature might increase the quantal release of neurotransmitters, thus increasing the amplitude of the synaptic events. The fact that GABA antagonists increased the frequency of the IPSPs might imply that GABAergic neurons are themselves under tonic inhibitory control. Even though paired recordings have not been attempted, it is very probable that the occurrence of IPSPs is correlated with the discharge of single pacemaker neurons. $GABA_A$ antagonists application produced, in addition, a membrane depolarization when neurons were recorded with KAc-filled electrodes. However, this effect needs to be interpreted with caution since a similar effect was produced in neurons recorded with KCl-filled electrodes (i.e. IPSPs were depolarizing). Even though it is probable that part of the depolarization is mediated by the suppression of a tonic inhibition, there could as well be non-specific effect on the $GABA_A$ blockers on the resting membrane potential.

Since GABAergic neurons are abundant in the RVL (see introduction), it is reasonable to assume that much of the GABA in the RVL is derived from intrinsic neuronal population. The depressor responses to baroreceptor activation [5,39,41] result from GABA-mediated inhibition of RVL sympathoexcitatory neurons. This inhibition could be mediated by GABAergic barosensory interneurons in the CVL different from the A1 noradrenergic neurons or alternatively by synaptic excitation of local GABAergic interneurons in the RVL [10,21]. Closely adjacent GABAergic RVL interneurons seem to mediate sympathoinhibition produced by medullary raphe nuclei stimulation [23]. Therefore, the source of GABAergic input to RVL neurons might be both endogenous and exogenous to the nucleus.

4.4. Identity of the pacemaker cells: sympathoexcitatory versus sympathoinhibitory function

It has been assumed that pacemaker neurons in the RVL maintain vasomotor tone via a reticulospinal sympathoexcitatory projection to the SPN in the intermediolateral cell column [45]. A spinal projection of some of the RVL pacemaker neurons has been demonstrated by the same authors; however, the identity of the neurotransmitter they synthesize is not yet known and there is no firm evidence that they are excitatory. Furthermore, clonidine, an effective antihypertensive sympatholytic agent, in concentrations up to 1 μM, did not alter the activity of the RVL pacemaker neurons sampled by Sun and Guyenet [42]. On the other hand, it was found that neither the pacemaker neurons recorded by Sun et al. [46] nor the RVL bulbospinal barosensitive neurons recorded intracellularly in vivo by Granata and Kitai [8] exhibited PNMT immunoreactivity, suggesting that C1 neurons are mostly silent.

Many electrophysiological studies support the existence of RVL GABAergic pacemaker neurons having a sympathoinhibitory function. It has been reported that when small injections of muscimol were used to inhibit the discharge of a limited population of neurons within the well-defined 'pressor' area of the RVL, an increase in sympathetic discharge and arterial pressure was sometimes observed. This disinhibition may have been caused by actions on tonically active GABAergic interneurons or bulbospinal sympathoinhibitory neurons distributed throughout the RVL [4]. The existence of a subpopulation of RVL sympathoinhibitory neurons has been proposed by

Li et al. [18] who found that some RVL units were excited by intravenous phenylephrine, thus responding in the opposite direction of the remaining RVL neurons. An effective inhibitory role of RVL on sympathetic activity has also been suggested by Poree and Schramm [30] who found that GABAergic inhibition of the RVL actually increases renal sympathetic discharge.

On the other hand, the hypothesis that RVL sympathoexcitatory neurons are either quiescent or have low basal discharge rate is supported by direct as well as indirect evidence. Recently, only neurons displaying these characteristics have been shown to be excited by angiotensin II and to be inhibited by the α_2-agonist UK-14,304, while the same drugs have no significant effect on PL rapidly firing neurons [19]. Spinal transection has been shown to increase sympathetic activity in some sympathetic nerves, suggesting that spinal systems are capable of generating sympathetic activity [47]. It has been also shown that a population of SPN neurons is spontaneously active and this may contribute to a large extent to the maintenance of the sympathetic tone independent of supraspinal structures [37].

4.5. Conclusion and physiological significance

Taken together, the present data suggest that IF neurons which have larger action potential duration, slower firing frequency or are sometimes quiescent, might be among the C1 adrenergic neurons that provide the sympathoexcitatory drive to SPN. However, their involvement in non-cardiovascular functions is also possible. At least some of the pacemaker neurons probably among the regular PL neurons act locally to provide tonic GABAergic inhibitory input to both GABAergic and non GABAergic neurons. Moreover, the difference in GABA responsiveness found for each group of neurons classified in this study might reflect functional difference in the mode of action of GABA depending on the physiological role of each of these groups of neurons. However, further electrophysiological and immunocytochemical studies are warranted to confirm these proposals.

Data obtained so far on RVL circuitry indicate that this nucleus is composed of both excitatory and inhibitory elements. Therefore, the cardiovascular effect of various chemicals microinjected into this brain region would be the resultant effect of all these elements. Additional complications result from the fact that some RVL pacemaker neurons have substantial widespread coronal dendrites [44] and the dendritic tree of some characterized bulbospinal C1 neurons extends beyond the boundaries of the RVL region [13]. Since the bulbospinal neurons constitute only a subpopulation of the total RVL neurons, all neurons in this vasomotor center might be equally important in mediating, directly or indirectly, the cardiovascular effects of potentially active drugs.

While glycine is strongly implicated in the intraspinal regulation of SPN activity [14], and baroreceptor control at the spinal site [17], it seems that GABA is the more likely inhibitory transmitter used by medullary structures to modulate, directly or indirectly, the sympathetic drive to the spinal cord.

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