Importance of Glycinergic and Glutamatergic Synapses Within the Rostral Ventrolateral Medulla for Blood Pressure Regulation in Conscious Rats

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Abstract—In this study we used a method that permits bilateral or unilateral microinjections of drugs into the rostral ventrolateral medulla (RVLM) of conscious, freely moving rats. There is only limited information about how sympathetic vasomotor tone is maintained by premotor RVLM neurons in conscious animals. It has long been known that glycine microinjection into the RVLM region leads to a decrease in blood pressure (BP) in anesthetized animals. In the present study we show that both unilateral and bilateral microinjection of glycine at the same dose used for anesthetized rats (50 nmol, 50 nL) into the RVLM increases BP in conscious animals. A similar response was also observed when the excitatory amino acid L-glutamate was microinjected into the RVLM. The microinjection of kynurenic acid into the RVLM did not change the basal level of BP but blocked the increase in BP after glycine or glutamate microinjection. A decrease in BP was only observed when low doses of glycine were used (1 to 10 nmol). We conclude that, in conscious animals, the hypertension occurring in response to high doses of glycine into the RVLM is dependent on glutamatergic synapses within the RVLM. A decrease in BP observed when low doses of glycine were used shows that in conscious animals, the RVLM, in association with other premotor neurons, is probably responsible for the maintenance of sympathetic vasomotor tone, because glycine is less effective in decreasing BP under these circumstances than in anesthetized animals. **(*Hypertension*. 1999;34[part 2]:752-755.)**

Key Words: vasomotor tone ■ ventrolateral medulla ■ glycine ■ hypertension, experimental ■ rats

The ventrolateral medulla (VLM) represents the central structure for the generation, maintenance, and reflex control of sympathetic vasomotor tone and pulsatile arterial blood pressure (BP).[1–3] Two distinct groups of neurons have been identified within the VLM: the rostral VLM (RVLM) contains sympathetic premotor neurons responsible for maintaining the tonic excitation of sympathetic preganglionic neurons involved in cardiovascular regulation,[1,2,4] and the caudal VLM is a depressor area clearly involved in reflex regulation of BP.[1,2] Recently, a third area, caudal to the caudal VLM, was identified in the VLM as a tonic caudal pressor area that contributes to maintaining basal levels of vasomotor tone and BP.[5,6] A significant proportion of tonic activity in RVLM sympathetic premotor neurons is driven by caudal pressor area neurons, at least in anesthetized rats.[6]

The RVLM is a pressor area containing sympathetic premotor neurons. There is a large body of evidence showing that the functional integrity of the RVLM is essential for the maintenance of basal vasomotor tone. Electrolytic lesion or chemical inactivation of RVLM neurons by inhibitory amino acids such as glycine or γ-aminobutyric acid results in a collapse of BP similar to that usually obtained in acute spinal animals.[3,7,8] Most of these studies were performed on anesthetized animals, a condition that may be a limiting factor in the interpretation and analysis of the results obtained. Conscious animals may be an entirely different entity because of the withdrawal of the anesthetic effects.

In the present study we examined the cardiovascular effects of different doses of glycine or glutamate microinjected into the RVLM of conscious, freely moving animals. The effects of glutamatergic synapse blockade on BP and on the cardiovascular responses to the microinjections were also tested.

Methods

General Procedures

Experiments were performed on male Wistar rats (weight, 300 to 350 g; n=48) from the Central Animal House of the Universidade Federal de São Paulo, Escola Paulista de Medicina in Brazil. All experiments were approved by the animal experimentation Ethics Committee of the Universidade Federal de São Paulo, Escola Paulista de Medicina. The procedure for drug microinjections into the RVLM of conscious animals has been described previously[9] and was a development of the method already used in our laboratory for acute experiments on anesthetized animals.[5,10]

Cannula Implantation into the RVLM

Three to 5 days before the experiments, animals were anesthetized with thiopental (40 mg/kg IP) and placed prone in a stereotaxic

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apparatus with the bite bar 5 mm below the interaural line. The lambda was taken as a landmark for the stereotaxic coordinates. To implant the guide cannulas, 2 occipital holes were bilaterally drilled caudal to the lambda, through which a stainless steel cannula was directed to the desired stereotaxic position in relation to lambda (anteroposterior = −2.2 mm, lateral = 1.8 mm).

Two separate electrode manipulators were used in the stereotaxic frame with independent movements (posterior inclination of 8°), allowing distinct anteroposterior and lateral measurements on each side. A stainless steel tube (OD = 1.5 mm, length = 15 mm) was fixed on the side of an electrode holder to contain and allow displacement of the support system of the guide cannula.

A stainless steel micropipette (OD = 0.3 mm) was placed inside the set of support and guide cannulas. The length of the micropipettes and the guide cannulas was adjusted to allow only the micropipettes to be inserted into the brain tissue. The guide cannula was then directed to the desired stereotaxic position. Vertical positioning was obtained by slowly lowering both the micropipette and the set of supporting and guide cannulas until a slight displacement between them was observed. Postmortem histology demonstrated that this procedure consistently permitted the placement of the micropipette tip juxtaposed to the surface of the ventral medulla, otherwise intact.

Two screws were placed 2 mm from the lambdoid sutures for further stabilization of the guide cannulas. Finally, the cannulas were surrounded by a ringlike crown structure to avoid animal contact. The animals recovered from surgery in a heat-controlled cage.

Twenty-four hours before the experiments, rats were anesthetized with ketamine (40 mg/kg IP) and xylazine (20 mg/kg IP), and the right femoral vein and artery were cannulated and dorsally exteriorized for drug infusion and for BP recording with the use of a transducer (Statham P23 Db) connected to a Beckman R511A recorder. Mean arterial pressure (MAP) was obtained by filtering the BP signal in a second channel, and heart rate (HR) was recorded with a cardiotachometer (Beckman 9857B) triggered by the pulse wave in a third channel. On the day of the experiment the animals were kept in their cages, and the basal recordings were obtained for at least 30 minutes.

Drug Microinjections

Drugs were microinjected into the RVLM through micropipettes placed inside the guide cannulas at positions determined previously, as described above, and connected to Hamilton (701) microsyringes (1 μL). Microinjections consisted of glycine (50, 10, or 1 nmol/50 nL), glutamate (10 nmol, 50 nL), kynurenic acid (4 nmol, 50 nL), or saline. The pH of the solutions was adjusted to 7.4. Injections into the RVLM were performed with the rats showing no external signs of discomfort or respiratory alterations.

Histology

At the end of the experiments, 50 nL of 2% Evans blue dye was injected into the ventromedullary sites. In some experiments the RVLM was marked with 1% horseradish peroxidase diluted in glycine. Animals were killed with an overdose of urethane, and the brain stem was removed and fixed in 10% formaldehyde for histological analysis. A characteristic injection site is shown in Figure 1.

Data Analysis

All values are expressed as mean±SEM. The significance of changes in MAP or HR after microinjection was determined within each group by Student's paired *t* test. Differences between groups were assessed by 1-way ANOVA followed by the Student-Newman-Keuls method. Differences were considered significant for a value of $P<0.05$.

Results

Effects of Bilateral or Unilateral Microinjection of High Doses of Glycine into the RVLM

Unilateral microinjection of glycine (50 nmol, 50 nL) into the RVLM provoked an increase in BP. In a group of 6 animals, unilateral injection of glycine into the RVLM significantly increased MAP (control, 117±3 mm Hg; after glycine, 155±7 mm Hg) (Figure 2A). The response started during the microinjection period, peaked at 1 minute, and remained above resting level for 3 minutes. A similar hypertension was observed after bilateral microinjection of glycine at the same dose (control, 114±3 mm Hg; after glycine, 150±5 mm Hg) (Figure 2B). The time course of the response was also similar to that observed in the unilateral injection. No significant change in HR was observed in either group. Microinjection of physiological saline alone (50 nL) did not produce any significant effect on basal MAP or HR (112±3 to 108±3 mm Hg and 305±21 to 300±21 bpm, respectively).

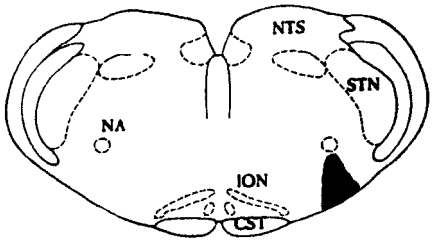

Figure 1. Schematic representation of an injection site evaluated by horseradish peroxidase deposit (shaded area) into the RVLM. NTS indicates nucleus of the tractus solitarius; STN, spinal trigeminal nucleus; NA, nucleus ambiguus; ION, inferior olivary nucleus; and CST, corticospinal tract.

Effects of Bilateral Microinjections of Low Doses of Glycine into the RVLM

When low doses of glycine were used, a decrease in BP was observed instead of the increase described above. Glycine microinjection (10 nmol, 50 nL) significantly decreased MAP (from 120±3 to mm Hg to 107±2 mm Hg), as shown in Figure 3A. The response started during the microinjection, peaked at 1 minute, and remained below resting levels for 4 minutes. A similar decrease was also observed when a lower dose of glycine (1 nmol, 50 nL) was microinjected (from 121±2 to 105±2 mm Hg), as shown in Figure 3B.

Effect of Glutamatergic Synapse Blockade Within the RLVM on the Hypertension Induced by Unilateral Glycine or L-Glutamate Microinjections

Unilateral microinjection of the broad-spectrum glutamate antagonist kynurenic acid into the RVLM did not change basal BP level but blocked the hypertension induced by high doses of both glycine and glutamate. Unilateral L-glutamate (10 nmol, 50 nL)

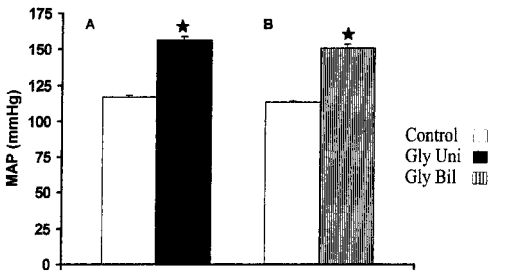

Figure 2. Maximal increase in MAP in response to unilateral (Uni) (n=6) or bilateral (Bil) (n=7) glycine (Gly) microinjection (50 nmol, 50 nL). *$P<0.05$.

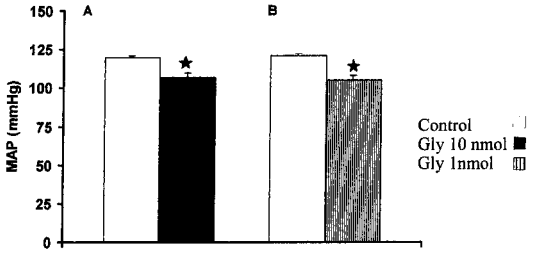

Figure 3. Maximal decrease in MAP in response to glycine (Gly) microinjected at a dose of 10 nmol (50 nL, n=6) or 1 nmol (50 nL, n=6). *$P<0.05$.

microinjection into the RVLM in freely moving rats produced an increase in MAP (from 117±2 to 156±7 mm Hg), as shown in Figure 4A. The response started during the microinjection, peaked at 1 minute, and remained above resting level for 2 minutes. After kynurenic acid microinjection, glutamate induced no significant change in MAP (from 117±3 to 115±2 mm Hg) (Figure 4B). This effective blockade lasted ≈60 minutes, and a full recovery was only observed after 3 hours. In another group of 7 animals, when glycine (50 nmol) was injected 15 minutes after kynurenic acid, a blockade of hypertension was observed (from 109±3 to 112±3 mm Hg), as shown in Figure 4C.

Discussion

The results of the present study demonstrate the following: (1) microinjection of glycine directly into the RVLM of conscious, freely moving rats elicited an increase in BP, particularly when a high dose (50 nmol) was used; (2) previous studies[9,11] showing that L-glutamate microinjected into the RVLM increased BP were confirmed; (3) the pressor responses to glycine or L-glutamate were blocked by prior injection of the broad-spectrum antagonist kynurenic acid into the RVLM; (4) a decrease in BP could be obtained only when low doses of glycine (1 to 10 nmol) were used; and (5) blockade of the glutamatergic synapses in the RLVM did not change the basal level of BP.

It is accepted that sympathetic premotor neurons of the RVLM are critical for the maintenance of ongoing excitatory drive that supports the activity of preganglionic vasomotor neurons.[1,2] Most of these studies were performed on anesthetized animals. When RVLM sympathetic premotor neurons were activated an increase in BP was observed,[10,12,13] and, more importantly, when they were inhibited BP fell to levels similar to those observed after acute spinal cord transection.[2,3,8,14] However, these studies were conducted on animals anesthetized with urethane, α-chloralose, or sodium pentobarbital, drugs with central and peripheral effects that might directly or indirectly modify the cardiovascular responses to RVLM inhibition. For example, Cochrane and Nathan[15] showed in rats that the hypotension produced by RVLM lesion depended on the anesthesia condition.

Although previous studies showed that chemical stimulation of RVLM neurons produced an increase in BP in conscious animals,[11] controversy has involved questions of the determination of vasomotor tone and the importance of RVLM activity in the maintenance of BP not only in anesthetized animals but also in conscious animals.

In the present study we found that the fall in BP caused by RVLM inhibition with low doses of glycine was less intensive than that observed in anesthetized animals. It is possible that in conscious animals other premotor neurons are capable of maintaining BP during RVLM inhibition by glycine. Particular nuclei in the brain stem and hypothalamus project directly to sympathetic premotor neurons such as the paraventricular nucleus, A5 noradrenergic cell group, and caudal raphe nuclei, which may therefore be involved in the maintenance of sympathetic vasomotor tone during withdrawal of RVLM neurons in conscious animals.[2]

Previous studies have demonstrated that glycine is a potential neurotransmitter in the VLM with a physiological significance, since it was shown that during baroreceptor stimulation or in response to potassium, glycine was released into the VLM.[16,17] It remains to be determined whether the glycinergic afferents in the RVLM are spontaneously active. In anesthetized cats, Guertzenstein[18] showed a dose-dependent increase in BP in response to strychnine applied into the RVLM. On the other hand, Ross et al[19] showed no change in BP or HR in response to strychnine microinjected into the same area in anesthetized rats. In addition to having a direct action on specific receptors, glycine can also induce release of other neurotransmitters such as acetylcholine.[20]

In studies on cats under chloralose anesthesia, Feldberg and Guertzenstein[21] demonstrated that an area exists in the medullary-spinal transition that appears to maintain BP in deeply anesthetized cats. These authors demonstrated that during surgical anesthesia with chloralose, the stimulation of this area with topical application of pentylenetetrazol produced a decrease in BP. In contrast, when the same animals were profoundly anesthetized, stimulation of this area produced an increase in BP, and its inhibition produced a decrease similar to that observed in response to inhibition of the RVLM. These experiments demonstrate the influence on and interference of anesthesia with the cardiovascular responses to pharmacological manipulation of the nervous system. Similarly, Bachelard et al[11] demonstrated that the cardiovascular responses to microinjection of L-glutamate into the RVLM are more prominent in conscious animals than in animals anesthetized with urethane.

One explanation for the observation of a different intensity of the cardiovascular response to microinjection of glycine into the RVLM of conscious or anesthetized animals could be that in conscious animals behavioral effects may cause secondary cardiovascular alterations that may mask the ac-

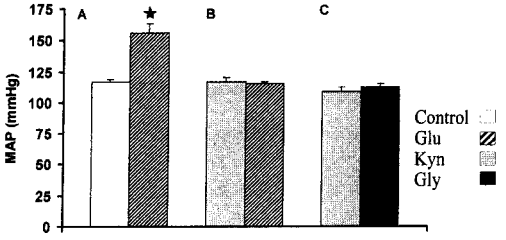

Figure 4. Maximal alterations in MAP in response to unilateral glutamate (Glu) microinjection into the RVLM (10 nmol, 50 nL, n=6) before and after kynurenic acid (Kyn) injection at the same region (4 nmol, 50 nL) or glycine (Gly) (50 nmol, 50 nL, n=6) microinjected unilaterally after kynurenic administration. *$P<0.05$.

tions of these drugs. However, this possibility seems to be remote since no behavioral alterations were observed in response to microinjections of glycine, L-glutamate, or kynurenic acid into the RVLM of conscious animals. Saline microinjections did not modify BP or HR, and no respiratory alterations were observed in response to the microinjections.

The RVLM exerts an excitatory influence on sympathetic vasomotor fibers, the adrenal medulla, and the posterior pituitary.[19] These neurons are tonically active, and the inhibition or activation of RVLM premotor neurons in conscious animals can change BP through these mechanisms.

Another important finding of the present work is that no change in BP was observed after kynurenic acid microinjection into the RVLM, showing that in the conscious condition the glutamatergic synapses within the RVLM are not important for maintenance basal BP. The same result was obtained previously in anesthetized animals.[5,22,23] Glutamatergic synapses in the RVLM seem to be important to maintain BP only in renovascular or spontaneously hypertensive rats.[10] Although no change in BP was observed after kynurenic acid microinjection, the acid totally blocked the hypertension induced by a high dose of glycine or glutamate. The hypertension induced by glycine can be explained by its effect at the *N*-methyl-D-aspartate receptor site.[24–26] It has been previously shown that glycine may potentiate the action of *N*-methyl-D-aspartate by acting on an allosteric site of the receptor and that kynurenic acid is capable of displacing glycine from this allosteric site.[24,27]

Finally, we would like to emphasize that although we have shown that inhibition of the RVLM produces a less effective reduction in BP in conscious, freely moving rats than in anesthetized rats, we cannot rule out the participation of this area in the maintenance of BP. It is conceivable that in conscious animals, the RVLM is responsible for the maintenance of vasomotor tone and sympathetic activity in association with other areas of the brain. During RVLM inhibition, these other areas outside the RVLM may assume its role in the maintenance of BP in conscious animals. An intense fall in BP could only be observed after sequential inhibition of different regions.

Acknowledgments

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