Neural and Cardiac Activities Are Altered by Injection of Picomoles of Glutamate into the Nucleus Ambiguus of the Rat

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Abstract

A quantitative evaluation of the thresholds of changes in the firing rate/pattern and depolarizing block of the neuron and the bradycardiac response by pressure microinjection of 10 mM glutamate (Glu) into the region of the nucleus ambiguus (NA) of the ventral medulla was performed in anesthetized rats. A change in neuronal activity was shown with injection of about 2 pmol of Glu. A depolarizing block of single-unit activity could be observed at 2.9 ± 0.3 nl (~30 pmol, *n* = 22). Maximal bradycardiac response (-50 ± 5%) was elicited with 4.4 ± 0.7 nl (~50 pmol, *n* = 10), which is significantly smaller than the ranges used in previous studies. Based on these results, a safe and effective use of 10 mM Glu to induce neuronal or physiological response should be in the range of a few nanoliters and less than 100 pmol, especially for the NA.

Key Words: microinjection, depolarizing block, nucleus ambiguus, heart rate, single-unit recording

Introduction

Anatomical and electrophysiological experiments have demonstrated in most mammalian species that the ventrolateral division of the loose and external formation of the nucleus ambiguus (NA) is the main site of origin of cardioinhibitory neurons as well as of axons to other visceral organs (see review, 10). Neurons in the NA are excited by stimulation of the carotid sinus nerve (3) and can be antidromically activated by electrical stimulation of the cardiac branches of the vagus (3, 12, 17). Several neurotransmitters, such as glutamate and glycine, have been shown in controlling the activity of NA neurons (2, 7, 14, 15). Furthermore, several areas, such as the nucleus tractus solitarius and rostral/caudal ventrolateral medulla, have been proposed to functionally interact with the NA (1, 14, 15). However, although the functional role of the NA in the control of the cardiovascular system has been established, detail operation of the neural network in the brain stem still remains largely unknown.

Several techniques have been used to address this question. Microinjection of specific chemicals into a brain locus is often the most practical way to reversibly activate/inactivate specific groups of neurons within the brain. Some excitatory amino acids have been shown to selectively activate almost all neurons, but not to affect axons of passage (6, 8, 19). Although the technique of pressure microinjection of excitatory amino acids is now used in a large number of laboratories (3, 4, 8, 11, 13, 23), the dose of excitatory amino acids used in each study, however, diverse from several to several hundred nanoliters (3, 4, 8, 9, 11, 13-15, 23). Previous studies have indicated that a higher amount of vehicle or excitatory amino acids (> 30 nl) will probably cause spurious effect due to local pressure change (5, 9, 21). Furthermore, a

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higher dose also induces a depolarizing block of local neurons around the injection site (6, 9). Because interpretation of results gained by pressure microinjection is based on the assumption that the evoked responses are due to a relatively long-lasting excitation of neurons located close to the injection site (8, 9), a depolarizing block of neurons in the local neural network will result in an improper interpretation. To comprehend the exact interaction within local neural circuits, an evaluation of the relationship between the injected dose and the evoked neuronal and physiological responses will be important, especially for the NA region here. A previous study has indicated that injection of picomoles of Glu into ventral respiratory group, including NA, is able to effectively alter the respiratory pattern via phrenic nerve recording (13). However, they did not monitor the neuronal activity at the injection site. In addition, it needs a higher amount (1 nmol in 10 nl) of Glu to elicit the cardiovascular effect in the similar area of the rat in some previous studies (2, 14, 15). What accounts for this difference in the same nucleus (NA) is worth to figure out. In this study, we measured the threshold doses required to elicit excitatory neural activity, depolarizing block of neuron, and bradycardiac response by picomole injection of 10 mM L-glutamate solution (Glu) in the NA via a recording-injection microelectrode. Comparing our results with those used in previous studies, a reasonable volume of Glu microinjection into the NA area is recommended for studying the operation of the neural network.

Materials and Methods

Experiments were performed in 20 adult male Wistar rats (250-350 g) anesthetized with 7.5% urethane and 1% α-chloralose (6 ml/kg, i.p. initially and 2 ml/kg, i.v. supplements as required). The femoral artery and vein were cannulated for measurement of systemic arterial pressure (SAP) and administration of drugs, respectively. Heart rate (HR) was online monitored through a tachometer (Grass 7P4G) triggered by the SAP pulse. The head of the rat was fixed in a Kopf stereotaxic apparatus. The dorsal medulla was exposed by retracting the dorsal neck muscles and incising the atlanto-occipital membrane. The medial-lateral and anterior-posterior coordinates of the obex were identified and used as a reference point for brain stimulation and unit recording. In addition, a lead II configuration of the electrocardiogram was also used to measure HR. A bipolar stainless steel electrode with fire-polished ball tips was pushed in until it abutted the diaphragm through an opening in the abdomen in order to record the diaphragm electromyogram (EMG) via a Grass amplifier (P511). Rectal temperature was maintained at 37 ± 0.5 °C with a thermostatically controlled heating blanket.

The injection-recording microelectrode was constructed with a commercial glass pipette (A-M systems: #6255) and a formvar-insulated nichrome microwire (A-M systems: #7610). The tips of both the microwire and micropipette were in the same plane. The tip of the micropipette was 10-50 μm in diameter. A JFET buffer headstage was used for reducing external interference. The entire setup including the neuronal amplifier was described in a previous study (20). The injection barrel was filled with a 10 mM Glu solution in artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$ and 11 mM glucose, pH 7.4) and 0.01% horseradish peroxidase (HRP), which served as a marker for identification of the injection site. This concentration of Glu is popularly used and has been confirmed to be able to evoke neuronal/physiological responses in several laboratories (4, 11, 13, 23). The Glu solution was injected with a pneumatic-pressure pump (Medical System, PPS-2). After the rat was adequately prepared, an injection-recording microelectrode was slowly advanced into the brain. On approaching the NA and/or when a single-unit activity appeared, Glu stimulations of different pulse durations were applied in a random order. The dose-dependent relationship between the evoked neuronal and bradycardiac responses and the ejected Glu volume was evaluated. The same injection duration was used at least 2 times to calculate the average evoked responses of neuronal activity and related physiological changes, such as bradycardia. The interval between microinjections was at least 5 min when the volume of Glu was larger than 1 nl. The injection volume was monitored by measuring the movement of the fluid meniscus (h) within the pipette barrel under the operating microscope equipped with a reticle in the eyepiece, then calculated by $\pi r^2 \times h$ (r: internal diameter of the barrel). An online interactive program, based on the LabVIEW environment (National Instrument, Austin, TX), was developed for extracting the R-peak and single-unit activity. Additional off-line analytic programs for cardiac and respiratory rhythmic tests and optimization of a logistic function were also established. All data are presented here as mean ± SE.

At the end of each experiment, the rat was sacrificed by intracardiac perfusion with saline followed by a 10% formalin saline solution. The brain was removed and blocked for serial frozen sectioning (50 μm) at the coronal plane. The sections were processed with the cobalt- and nickel- intensified diaminobenzidine (DAB) method for the histochemical demonstration of HRP deposits and

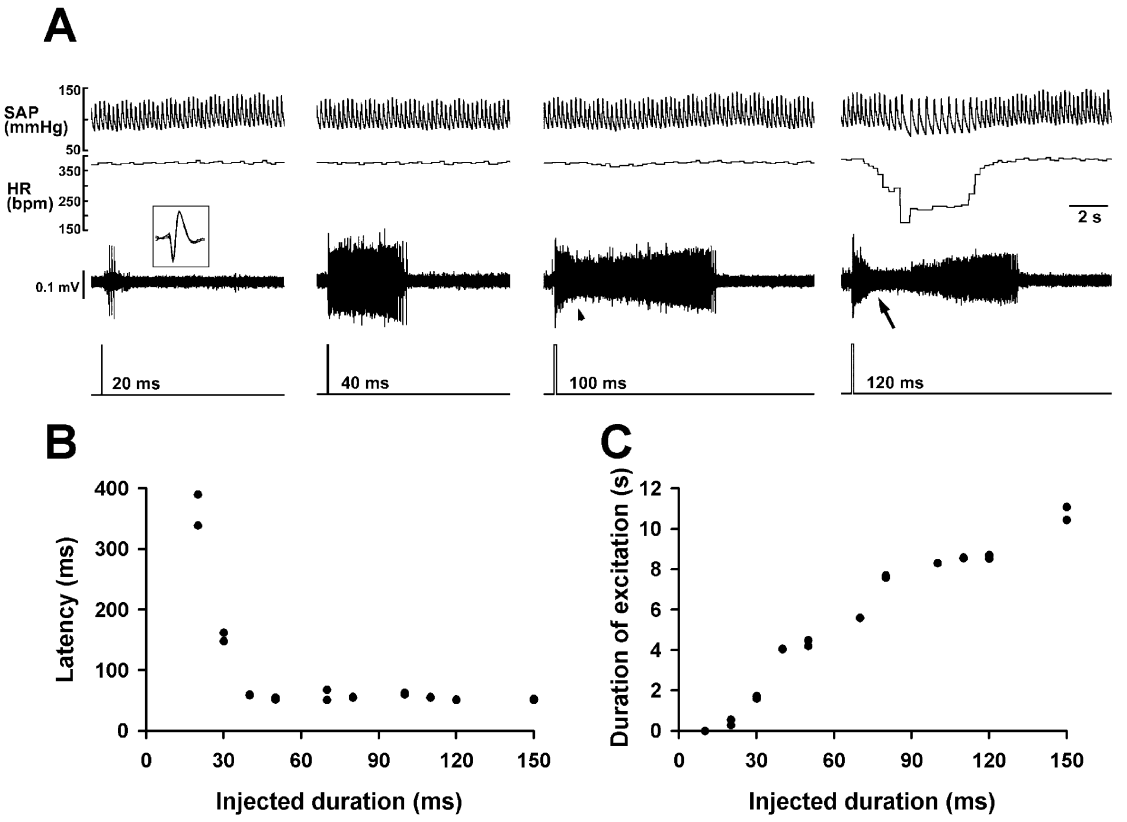

Fig. 1. A representative example of NA unit and heart rate responses to various amounts of Glu stimulation. (A) High-amplitude and small neural activities coexcited by ~ 0.2 nl of Glu in the leftmost column. The three single-unit potentials are expanded and shown in the inset. Pulse durations of 40, 100, and 120 ms produced ejection volumes of 1, 2, and 4 nl, respectively. An amplitude decrease in unit activity is shown at 100-ms injection duration (arrowhead). A possible depolarizing block of NA neurons was observed at higher doses (arrow). The threshold of the depolarizing block of this neuron is about 3.5 nl. A large bradycardiac response (-55%) can be seen in the rightmost panel under a pulse duration of 120 ms. SAP: systemic arterial pressure; HR: heart rate. (B) Latency of the evoked neuronal activity decreased as the injected duration of Glu increased. Latency was calculated from the difference between the beginning of the pressure pulse and the time of the first spike of the neuron. (C) Duration of excitation of the evoked neural activity lengthened as the injected duration of Glu increased.

counterstained with thionin to verify the stimulation site.

Results

An example of the change of neuronal activities and the bradycardiac effect elicited by Glu stimulation of the NA region is shown in figure 1. A neuronal response elicited by Glu could be observed with a very small amount (~ 0.2 nl as calculated from the cumulative measure of 50 repetitive injections). The averaged threshold of neuronal activity was smaller than 2 pmol (n = 22). The neuronal responses to Glu stimulation increased with increases in the injected volume (Fig. 1). An increase in the injected volume of Glu resulted in a shortening of the responsive latency and a lengthening of the neuronal response (Fig. 1B, C). The phenomenon of a neural depolarizing block, which can be seen as a reversible abolishing of the spikes (arrow in Fig. 1), was always observed in all recording trials with higher Glu doses. Another example of an inspiratory-related neuron in the NA area is shown in Fig. 2. A clear neuronal response was elicited with a small amount of Glu (leftmost panel of Fig. 2). By contrast, a large amount of Glu injection caused a depolarizing block, which is indicated by an extinction of the rhythmic neuronal activity of the inspiratory-related neuron (arrows in Fig. 2). To quantitatively evaluate the phenomenon of the depolarizing block of a single-unit, we measured the smallest volume of Glu, which elicited an obvious transient cessation of the evoked neuronal activity, as the threshold of the depolarizing block of the neuron. The threshold for neural depolarizing block fell in the range of 1.5-7.5 nl (2.9 ± 0.3 nl, n = 22).

On the other hand, a HR decrease following Glu stimulation was usually accompanied with long-train spike activity (rightmost panel in Fig. 1). In this study, we selected the site that produced the maximal bradycardiac response in each individual rat to

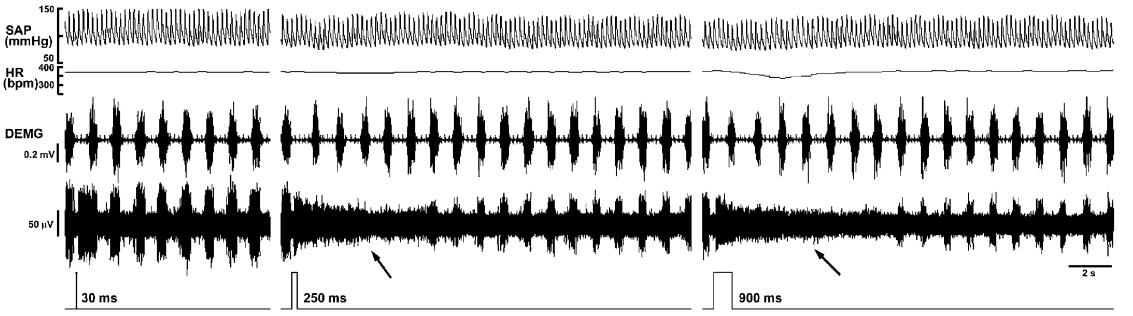

Fig. 2. A representative example of inspiratory-related unit activity around the NA is shown and its responses to various amounts of Glu stimulation. Pulse durations of 250 and 900 ms produced ejection volumes of 2.5 and 7.5 nl, respectively. Increased firing rate of this inspiratory-related neuron was elicited by a small injection of Glu under a pulse duration of 30 ms. The respiratory rhythm of the unit was not changed. A depolarizing block of the neuron is seen at higher doses (arrows). A small bradycardia (-27 bpm, -7.3%) is depicted in the rightmost column under a pulse duration of 900 ms. The threshold of the depolarizing block of this inspiratory-related neuron is about 1.75 nl. DEMG: diaphragm EMG.

represent the Glu-induced response of the NA. In addition, these sites must also be verified by HRP deposits to be in the vicinity of the NA. Specifically, a pure bradycardiac site (n = 10) is defined as the site where a dose-HR response curve can clearly be seen (Fig. 3A), without significant changes in either respiratory patterns or blood pressure. These sites were collectively shown on a reconstructed brain map (Fig. 3B). The HR baseline value was 367 ± 10 bpm (beats per minute). A logistic function was used to estimate the dose-dependent relationship between the evoked bradycardiac response and the injected Glu volume. The average threshold to generate a bradycardiac response by Glu stimulation was 1.8 ± 0.2 nl (~ 20 pmol), which is ten times larger than that of evoked neuronal activity. The average dose to produce the maximal bradycardiac response (-50 ± 5%) elicited by Glu was 4.4 ± 0.7 nl (~ 50 pmol). The maximal bradycardiac responses were not significantly different (P > 0.05) between left and right NA regions. Quantitative data on Glu volumes for threshold and maximal response of NA neural activity and the change of HR are summarized in Table 1.

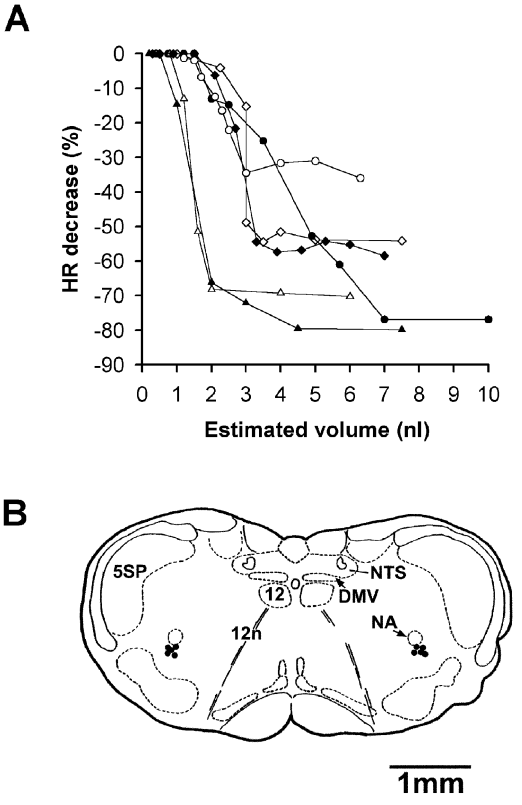

Fig. 3. (A) Dose-response curves of injection volume (Glu) vs. HR decrease. Six randomly selected examples illustrate the sigmoid relationship between the HR decrease and the injected Glu volume. HR decreases (%) were normalized with respect to the pre-stimulation HR value. Each point in the curve represents an average value of two injections. (B) Distribution of cardioinhibitory sites in the medulla where the maximal bradycardiac responses were elicited by Glu injections. These sites in ten animals, distributed from 0.3 to 0.7 mm caudal to the obex, were collectively reconstructed on the representative section. Abbreviations: DMV, dorsal motor nucleus of the vagus; NA, nucleus ambiguus; NTS, nucleus tractus solitarius; 5SP, spinal nucleus of trigeminal nerve; 12, nucleus of the hypoglossal nerve; 12n, roots of the hypoglossal nerve.

Discussion

Microinjection or iontophoresis of Glu produces several effects on unit activity: an increase in the discharge rate, a progressive increase in discharge duration, and a decrease in amplitude of the action potential (6). These phenomena were consistently observed in our experiments (Fig. 1 and 2; 20). A clear neuronal response could be seen after an injection of < 2 pmol Glu here as well as in our previous study (20). The threshold amount for excitation of neurons by Glu has been pointed out to be approximately 10^{-14} mol in theory (18). The theoretical value is

Table 1. Amounts of 10 mM Glu Stimulation to Elicit NA Neuronal Activity and Change of Heart Rate (Bradycardia) and to Obtain Their Maximal Responses

Glu volume	NA neuronal activity	Change of heart rate
Threshold	< 0.2 nl (~ 2 pmol)	1.8 ± 0.2 nl (~ 20 pmol)
Maximum	2.9 ± 0.3 nl (~ 30 pmol)[a]	4.4 ± 0.7 nl (~50 pmol)

[a]Threshold for depolarizing block.

smaller than our result. However, this threshold value is influenced by several factors, such as the density and distribution of receptors of neurons in different regions. A previous study observed that large changes in respiratory and/or cardiovascular neuronal discharges are elicited by medullary injections of 200 fmol to 10 pmol Glu (13). Our observation (2 pmol) is in the same range.

With pressure microinjection, the initial concentration of Glu is much higher at short distances because initial concentrations cannot be quickly reduced due to saturation of active uptake mechanisms. That causes the rapid development of a depolarizing block. In this study, the threshold amount to generate a depolarizing block of a neuron was measured as 30 pmol. Compared with the suggested amount (5 nmol) in a previous study (9), our data is at least 100 times smaller. This significant difference might primarily be the result of different experimental setups. A larger injected volume (30 nl) and a longer inter-electrode distance (250-500 μm) were used in the previous study (9). Because of the longer distance between the recording and the injecting electrodes, a higher amount of Glu would be needed to activate neurons located several hundred micrometers away. Thus a large neuron population will be activated under that preparation. By contrast, alternative volumes and tight binding of the recording-injection microelectrode pairs were used here. A smaller amount of Glu was sufficient to evoke the neuronal activity. In general, recording local neuronal activity around an injection site is essential for understanding the operation of local neural circuitry and evoked behavioral changes. Therefore, evaluating changes in local neuronal activity with our setup is more appropriate. It is our conclusion that the depolarizing block of a neuron will occur with an injection of ~ 30 pmol Glu (on average).

The threshold dose of Glu in generating a depolarizing block varied a lot (15-75 pmol) for a given neuron in this study. This threshold dose depends on several parameters, for example, the rate of the uptake mechanisms, the distance between the cell and the injection micropipette, the cytoarchitecture of the nucleus, etc. These factors will cause variations in the threshold of the neural depolarizing block. Nevertheless, based on our observations of 22 neurons in the NA region, the threshold doses were smaller than 100 pmol. Thus, a safe volume to avoid depolarizing blocks of a local neuron by Glu stimulation should be in the picomoles range, instead of 5 nmol (9).

In the present study, the effective amount to obtain a maximal bradycardiac response by Glu stimulation at NA was about 50 pmol (5 nl). This amount is significantly smaller than that used in previous studies (4, 8, 14, 15). Lipski et al. (1988) indicated that injection volumes should be smaller than 30 nl to avoid a non-pharmacological neuronal response (5, 9, 21). Our observation falls in a safe range. On the other hand, the initial sphere formed by 5 nl solution is about 106 μm in radius, and the theoretical effective radius of spreading is about 225 μm (16), which is sufficient to strongly impact the NA area. Injectate of 30 nl will affect an even greater area. Too large of an activated area will easily elicit complex effects, for example, a hyperpolarizing action on some neurons by Glu stimulation (22) and/or intricate postsynaptic responses induced by the release of local neurotransmitters of local terminals (18). In addition, a large amount of Glu will also activate neurons in the region nearby, such as the ventrolateral medulla (VLM). The ventrolateral medulla influences cardiovascular function via sympathetic outflow (14, 15). That will be the same (caudal VLM) with /or contrary (rostral VLM) to the cardioinhibitory effect of NA stimulation. Therefore the results will be difficult to interpret, or the response will be relatively attenuated. From physiological evidence, the bradycardiac response (-50%) evoked by 5nl (50 pmol) Glu is greater than that (about -25%) evoked by at least 10 nl (1 nmol) Glu stimulation in other studies (Table 1; 2, 14, 15). A different activated territory might account for this difference. Several reports have indicated that a dramatic bradycardiac effect (about -50%) can be elicited by a small amount (100 pmol) of Glu in the nucleus tractus solitarius (11). In addition, a comparable picomolar range of Glu injection into NA region could also alter the respiratory pattern (13). These data support our observation that a small amount of Glu is sufficient to modify physiological function. Based on these results, a safe

and effective use of Glu to induce neuronal or physiological response should be in the range of a few nanoliters and less than 100 pmol in the NA region.

Physiological behavior can be modified by the temporal and spatial summation of neuronal activity. However, most studies do not simultaneously monitor in situ neuronal activity and physiological changes as Glu injection (2,13-15). The quantitative relation between evoked neuronal response and behavioral changes is not yet established. To fill the gap, we recorded both activities simultaneously in this study and observed that there was an interesting relationship between different Glu volumes and the NA neuronal activity and cardiac response (Table 1). The Glu volume of the threshold of the bradycardiac response (~ 20 pmol) was smaller than that of the depolarizing block of NA neurons (~ 30 pmol). In other words, the change in heart beat induced by Glu occurs before the formation of a depolarizing block of NA neurons on average. A 20-pmol Glu stimulation will lengthen the responsive duration of the neuron (Fig. 1A), and consequently, will build up a temporal summation of neuronal activity to decrease HR. In addition, a higher amount of Glu will simultaneously activate more neurons, which may develop a spatial summation of neuronal activity. Combining the spatial and temporal summation of activity from a neuron pool may produce the maximal changes of heart rate. Thus an obvious physiological change (bradycardia) might be obtained by stimulating a large neuron population rather than a single neuron with the pressure microinjection of Glu.

In summary, a safe and effective use of Glu to induce neuronal or physiological responses should be in the range of a few nanoliters and less than 100 pmol. Detailed values are depicted in Table 1. These data should be useful not only for investigating the operation of the local neural circuitry of the NA, but also for the study of the function of other brain nuclei.

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