Responses to GABA$_A$ Receptor Activation Are Altered in NTS Neurons Isolated From Renal-Wrap Hypertensive Rats

Gleb Tolstykh, Sergei Belugin, Olga Tolstykh, Steve Mifflin

Abstract—The inhibitory amino acid GABA is a potent modulator of the spontaneous discharge and the responses to afferent inputs of neurons in the nucleus of the solitary tract (NTS). To determine if responses to activation of GABA$_A$ receptors are altered in hypertension, GABA$_A$ receptor-evoked whole cell currents were measured in enzymatically dispersed NTS neurons from 33 normotensive (NT, 109±4 mm Hg, n=7) and 24 hypertensive (HT, 167±5 mm Hg, n=24) rats. GABA$_A$ receptor–evoked currents reversed at the calculated equilibrium potential for chloride and were blocked by bicuculline (n=6). Membrane capacitance was the same in neurons from NT (7.5±0.6 pF, n=62) and HT (6.8±0.6 pF, n=51) rats. The EC$_{50}$ for peak GABA-evoked currents cells was significantly greater in neurons from HT (21.0±2.6 μmol/L, n=16) compared with NT rats (13.0±1.8 μmol/L, n=14, P=0.01). The EC$_{50}$ of neurons exhibiting DiA labeling of presumptive aortic nerve terminals was no different than that observed in the nonlabeled cells (19.0±4.9 μmol/L, n=4). The time constant for desensitization of GABA$_A$-evoked currents was the same in neurons from HT (4.5±0.3 seconds, n=17) and NT rats (3.8±0.3 seconds, n=17, P>0.05). Repetitive pulse application of GABA revealed a more rapid decline in the evoked current in neurons from HT compared with NT rats. The amplitude of the 5th pulse of GABA (5-second duration, 2-second interval) was 21±2% the amplitude of the 1st pulse in NT rats (n=10) and 14±2% in HT rats (n=11, P<0.05). These alterations in GABA$_A$-receptor evoked currents could render the neurons less sensitive to GABA$_A$ receptor inhibition and influence afferent integration by NTS neurons in HT. (Hypertension. 2003;42[part 2]:732-736.)

Key Words: amino acid ■ baroreceptors ■ baroreflex ■ phosphorylation ■ hypertension, renal ■ neuroregulators

A variety of experimental approaches have established the importance within the nucleus of the solitary tract (NTS) of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) as a modulator of cardiovascular and respiratory function. GABA$_A$ receptors are the ubiquitous ligand-gated chloride ionophores responsible for most rapid inhibition within the central nervous system. Within the NTS, activation of GABA$_A$ receptors inhibits virtually all neurons tested and results in an increase in arterial pressure, heart rate, and sympathetic outflow, effects consistent with inhibitory modulation of arterial baroreceptor reflexes. The function of GABA$_A$ receptors has been shown to be modulated by a variety of factors. Receptor number, subunit composition, and phosphorylation state are but a few of the ways that GABA$_A$ receptor function can be altered after chronic exposure to alcohol, barbiturates, benzodiazepines, and GABA itself. Alterations in the responses of NTS neurons to GABA$_A$ receptor activation could result in alterations in the integration of afferent inputs, reflex function, and resting cardiovascular parameters. The goal of the current study was to determine if the responses of isolated NTS neurons to activation of GABA$_A$ receptors are altered in hypertension.

Methods

General

Successful experiments were performed on adult (1 to 3 months), male Sprague-Dawley rats (375 to 500 g, Charles River Laboratories or Harlan Sprague-Dawley Inc). Rats were housed 2 per cage in a fully accredited (Association for Assessment and Accreditation of Laboratory Animal Care and the United States Department of Agriculture) laboratory animal room with free access to food and water. All rats were given at least 1 week to acclimate before being used for any procedures. The Institutional Animal Care and Use Committee approved all experimental protocols.

Chronic Hypertensive Model

Hypertension was induced using a 1-kidney renal-wrap procedure. Rats were anesthetized with medetomidine (0.5 mg/kg IP, Pfizer) and ketamine (75 mg/kg IP, Fort Dodge Laboratory). A figure-8 Grollman renal wrap and contralateral nephrectomy were performed on these animals. Control animals were sham-operated rats that were similarly anesthetized and received a unilateral nephrectomy but no wrap of the contralateral kidney or rats with no surgical procedures before the day of the experiment. Since the responses of both groups of normotensive rats were identical, they were grouped together for analysis. Anesthesia was terminated by atipamezole (1 mg/kg IP, Pfizer) at the conclusion of the surgical procedures. Postoperative analgesics (Nubaine, given intramuscularly) were available as needed. In 4 rats, a second surgery was performed...
weeks after the first, and under the previously described anesthetic regimen and aseptic conditions, the anterograde fluorescent tracer DiA was applied to the aortic nerve to visualize baroreceptor synaptic terminals and neurons receiving these synaptic contacts as previously described. 6

Dispersion of Caudal NTS Neurons

Experiments were performed 4 to 6 weeks after renal wrap or sham surgery. Two days before the experiment, an arterial catheter was placed in the femoral artery while the animal was under medetomidine/ketamine anesthesia (0.5 mg/kg IP and 75 mg/kg IP, respectively). After a 2-day recovery period, the blood pressure of the conscious animal was measured by connecting the arterial catheter to a pressure transducer (Kobe) and displayed by means of MacLab or Cambridge Electronics Design A/D converters. Blood pressure was measured for 3 hours, and measurements made during the last hour were used as an index of mean arterial pressure (MAP). Conscious blood pressure was measured in every hypertensive (HT) rat (n=24) to ensure the rat was indeed HT. Conscious MAP was measured in 7 of 33 normotensive (NT) rats. The properties of cells obtained from NT rats in which MAP was measured were no different from those of cells in rats in which MAP was not measured therefore the data were pooled for analysis. All EC50 measurements were obtained from cells in rats with measured MAPs. Rats were anesthetized with isoflurane and the brain stem was rapidly removed and placed in ice-cold Krebs buffer. The brain stem was cut into 440-μm-thick transversal sections centered around calamus, with the use of a vibratome (Warner Instrument Corp). The sections were incubated in the PIPES buffer with trypsin (Sigma, type XI, 3 to 7 mg/10 mL) for 60 minutes (Warner Instrument Corp). The sections were incubated in the PIPES buffer with trypsin (Sigma, type XI, 3 to 7 mg/10 mL) for 60 minutes (Warner Instrument Corp). Fast application of GABA is critical as the response to GABA begins to desensitize quite rapidly. Each pipette was attached by tubing to separate valves connecting each pipette to up to 6 solution ports, using a fast-step perfusion apparatus (Warner Instrument Corp). Fast application of GABA is critical as the response to GABA begins to desensitize quite rapidly. Each pipette was attached by tubing to separate valves connecting each pipette to up to 6 solution ports, using a fast-step perfusion apparatus (Warner Instrument Corp). Fast application of GABA is critical as the response to GABA begins to desensitize quite rapidly. Each pipette was attached by tubing to separate valves connecting each pipette to up to 6 solution ports, using a fast-step perfusion apparatus (Warner Instrument Corp). Fast application of GABA is critical as the response to GABA begins to desensitize quite rapidly. Each pipette was attached by tubing to separate valves connecting each pipette to up to 6 solution ports, using a fast-step perfusion apparatus (Warner Instrument Corp). Fast application of GABA is critical as the response to GABA begins to desensitize quite rapidly. Each pipette was attached by tubing to separate valves connecting each pipette to up to 6 solution ports, using a fast-step perfusion apparatus (Warner Instrument Corp). Fast application of GABA is critical as the response to...
Data Analysis
Sequential dose-response relations were obtained during 5-second applications of 1, 3, 10, 30, 100, and 300 μmol/L GABA, with at least 2-minute intervals between all drug applications. Curves were fit using

\[ I = I_{\text{max}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + \left( \frac{A}{EC_{50}} \right)^n} \]

where \( I \) is the GABA-induced current, \( I_{\text{max}} \) the maximal GABA current, \( I_{\text{min}} \) the minimal GABA current, \( A \) the concentration of GABA, and \( n \) the Hill slope. Curve fits were obtained by a nonlinear regression, using a 4-parameter logistic function. Desensitization was observed during application of 10 μmol/L GABA, and a time constant describing the process was obtained by means of a single exponential fit. Statistical significance of differences between NTS neurons obtained from normotensive and hypertensive rats was determined by means of ANOVA with the Tukey or Dunn tests used for post hoc comparisons. All values are expressed as mean±SEM, and significance was accepted at \( P<0.05 \).

Results
Data were obtained from 33 normotensive (NT, MAP 109±4 mm Hg, \( n=7 \)) and 24 hypertensive (HT, MAP 167±5 mm Hg) rats (\( P<0.001 \)). Examples of dispersed NTS neurons are provided in Figure 1. The membrane capacitance of neurons provides a rough index of cell size, and there was no difference in neurons obtained from NT (7.52±0.55 pf, \( n=62 \)) compared with HT (6.84±0.65 pf, \( n=51, P>0.05 \)). There was no difference between neurons from NT and HT rats in the reversal potential of the GABA_A receptor–evoked current determined by I-V curves obtained during application of 10 μmol/L GABA (NT, 0.3±1 mV, \( n=6 \); HT, −1±0.6 mV, \( n=4 \); calculated reversal potential for chloride based on bath and pipette compositions was −0.7 mV, Figure 2A). GABA_A receptor–evoked currents evoked by 3 μmol/L GABA were completely abolished after application of 30 μmol/L bicuculline (\( n=6 \)) (Figure 2B).

Dose-Response Relations
Increasing concentrations of GABA were sequentially applied at 2- to 3-minute intervals (Figure 1B). At concentrations >10 μmol/L, there was a noticeable decay in the peak current despite continued application of GABA. At least 2 minutes was allowed to elapse between each 5-second application to minimize the effects of this desensitization that...
developed during one application from influencing the response to a subsequent application. Sequential application of increasing GABA concentrations revealed a rightward shift in the GABA dose-response curve in HT rats measured at the peak of the response and 5 seconds later (Figure 3). The EC50 of the peak current density evoked by GABA was $13.03 \pm 1.75 \mu$mol/L in neurons from NT rats ($n=14$) and $21.03 \pm 2.63 \mu$mol/L in neurons from HT rats ($n=16$, $P=0.02$) (Figure 3A). This difference was even more pronounced when examining GABA-evoked currents 5 seconds after the onset of application (Figure 3B); however, the shape of the curve did not permit fitting to derive an “adapted” EC50.

The dose-response curves obtained in cells from HT rats exhibiting DiA labeling were no different from the rest of the HT population (EC50, $19 \pm 5 \mu$mol/L, $n=4$).

The decay of GABA-evoked currents at concentrations $>10 \mu$mol/L indicates desensitization of GABA-evoked currents occurs in neurons from NT and HT rats. Alterations in desensitization could lead to a shift in EC50; therefore, we looked at this process in more detail.

**Time-Dependent Changes in GABA Receptor–Evoked Responses**

Previous studies have reported time-dependent reductions in GABA currents with time due to depletion of intracellular ATP.9 To determine if run-down of GABA-evoked currents contributed to the differences in EC50 between NT and HT rats, GABA was applied (1 mmol/L, 2-second duration) at the onset of a recording and every 5 minutes for 25 minutes. The peak current density after 25 minutes of recording was $94 \pm 11\%$ of the value obtained at the onset of the recording in NT rats ($n=8$) and $95 \pm 3\%$ in HT rats ($n=3$, $P>0.05$). Desensitization was examined during 30-second applications of 100 μmol/L GABA. A single exponential curve fit of the decay in current from peak amplitude revealed no difference in the time constant of desensitization, comparing neurons from NT rats (3830±339 ms, $n=17$) with neurons from HT rats (4514±298 ms, $n=17$, Figure 4A). There was also no difference in the peak current density response to 100 μmol/L GABA of neurons from NT rats (419±51 pA/pf, $n=26$) with neurons from HT rats (443±41 pA/pf, $n=26$).

To examine responses to repetitive applications of GABA, five 5-second pulses of 1 mmol/L GABA were applied at 2-second intervals (Figure 4B). There was a significant reduction in the GABA-evoked currents during the 4th and 5th pulses in neurons from HT rats. In NT rats, the peak current amplitude of the 5th pulse was $21 \pm 2\%$ ($n=10$) of that measured during the 1st pulse. In HT rats, the amplitude of the 5th pulse was $14 \pm 2\%$ ($n=11$, $P<0.05$) that of the 1st pulse.
Discussion
These results demonstrate that the responses of NTS neurons to activation of GABA\textsubscript{A} receptors are reduced in HT rats. Activation of GABA\textsubscript{A} receptors inhibits NTS neurons receiving arterial baroreceptor, and most other visceral afferent inputs and activation of GABA\textsubscript{A} receptors during visceral afferent stimulation have been described.\textsuperscript{1} A reduction of this inhibition in HT rats in the absence of any other changes would be expected to enhance excitatory transmission through the NTS.

An important question is if the changes observed are specific for neurons receiving baroreceptor inputs. The identification of neurons with somatic DiA appositions is suggestive but not demonstrative. The answer to this question can best be answered by identification of the stimulus that initiates reduced sensitivity to GABA and this has yet to be determined. It is possible that excitatory and/or inhibitory synaptic inputs from peripheral or central sites could alter GABA\textsubscript{A} receptor function. It is also possible that circulating hormones associated with HT (catecholamines, angiotensin II) might initiate the alterations. The precise mechanism(s) that initiate and sustain the alterations are an important determinant of the specificity of the observed changes. If the stimulus is a specific afferent input, one would predict that changes occur only in those neurons receiving the afferent input; whereas if the stimulus is a hormone, alterations in GABA\textsubscript{A} receptor–evoked responses may be observed in a more widespread population of neurons. The present results suggest but do not conclusively prove the latter possibility.

Mechanisms for reduced sensitivity to GABA are as numerous as are the possible triggers for such changes. In response to other perturbations, GABA\textsubscript{A} receptors have been shown to undergo changes in subunit composition, changes in the number and/or affinity of binding sites, and changes in intracellular calcium and/or phosphorylation state leading to enhanced or reduced conductance.\textsuperscript{3,4} All of these possibilities remain avenues for future studies.

Although our data indicate that desensitization during a single application of GABA was not different in neurons from NT compared with HT rats, there was a difference in the responses to repetitive application of GABA. There are several possible explanations for this observation. A reduced ability to recover from desensitization,\textsuperscript{10} an alteration in the chloride equilibrium potential and/or altered activity of chloride exchange mechanisms,\textsuperscript{11} and alterations in phosphorylation state\textsuperscript{12,13} could contribute to what appears to be a cumulative effect of brief GABA applications. The functional significance of this observation may be manifested when the GABAergic inputs are pulsatile as opposed to tonic in nature.

Perspectives
NTS neurons are less sensitive to GABA\textsubscript{A} receptor–mediated inhibition in HT. In the absence of any other changes, reduced GABAergic inhibition of NTS neurons would lead to increased spontaneous discharge and enhanced responses to activation of afferent inputs.\textsuperscript{1} However, we have previously demonstrated in this model of HT that NTS neurons receiving aortic baroreceptor afferent inputs exhibit normal discharge frequencies and responses to aortic nerve stimulation.\textsuperscript{14} We have also demonstrated enhanced responses to activation of GABA\textsubscript{B} receptors in this model of HT,\textsuperscript{15} and this may mitigate the consequences of reduced GABA\textsubscript{A}-mediated inhibition.

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