ABSTRACT

Single units in the region of the medial nucleus tractus solitarius (NTS), responding to electrical stimulation of gastric vagal fibers, were recorded in an \textit{in vitro} neonatal rat brainstem-gastric preparation. \textgamma-Aminobutyric acid (GABA) subreceptor agonists and antagonists were applied to the gastric and brainstem compartments of the bath chamber to evaluate the peripheral gastric and central brainstem GABAergic effects on NTS neuronal activity. The gastric effects of the GABA\textsubscript{A} receptor agonist muscimol and GABA\textsubscript{B} receptor agonist baclofen were evaluated on 55 tonic units that received the gastric vagal inputs. For \textasciitilde 58\% (32 of 55) and 38\% (21 of 55) of the units observed, muscimol (30 \textmu M; IC\textsubscript{50} = 2.0 \textmu M) and baclofen (30 \textmu M; IC\textsubscript{50} = 1.5 \textmu M) in the gastric compartment induced a concentration-dependent inhibition of 36.2 \pm 3.1\% (mean \pm S.E.) and 31.0 \pm 2.9\% of the control level of the NTS neuronal activity, respectively. The brainstem effects of muscimol and baclofen were tested on 51 units. For \textasciitilde 90\% (46 of 51) and 78\% (40 of 51) of the units tested, muscimol (30 \textmu M; IC\textsubscript{50} = 1.3 \mu M) and baclofen (30 \textmu M; IC\textsubscript{50} = 1.1 \mu M) in the brainstem compartment produced a concentration-dependent inhibition of 54.1 \pm 3.4\% and 48.9 \pm 3.5\% of the control level, respectively. The remaining NTS units were not affected by these two GABA agonists. Bicuculline (10 \mu M) and saclofen (10 \mu M), the GABA\textsubscript{A} and GABA\textsubscript{B} subreceptor antagonists, competitively antagonized the gastric and brainstem effects by muscimol and baclofen, respectively. Our results demonstrated that both GABA\textsubscript{A} and GABA\textsubscript{B} receptors in the stomach and brainstem play an important role in activity modulation of the medial NTS neurons receiving gastric vagal inputs in neonatal rats.

The NTS is the primary brainstem relay for visceroreceptive information from the cardiovascular, respiratory, gastrointestinal and taste systems. A number of neurochemicals in the NTS are critical in coding visceral information (Maley, 1996). In our previous study, using an \textit{in vitro} neonatal rat brainstem-gastric preparation with intact vagi, we demonstrated that gastric \textmu and kappa opioid receptor agonists had significant inhibitory effects on NTS neuronal activity, suggesting that the gastric opioid receptors play a role in regulating the digestive process (Yuan, 1996). Subsequently, using the same preparation, we observed that after substance P application to the gastric and brainstem compartments, there was concentration-related activation of the NTS unitary activity (Yuan and Lowell, 1997).

The NTS is abundant in neuroactive substances, including GABA (Maley, 1996). GABA is an important inhibitory neurotransmitter that mediates the vertebrate central nervous system (Bormann and Feigenspan, 1995). Two major subtypes of GABA receptors are well known. GABA\textsubscript{A} receptors are ligand-gated Cl\textsuperscript{\textminus} channels that consist of a heteromeric mixture of protein subunits forming a pentameric structure. GABA\textsubscript{B} receptors couple to Ca\textsuperscript{\textplus} and K\textsuperscript{\textminus} channels via G proteins and second messengers (Johnston, 1996). The GABA receptors are present in the medullary pathway of mechanoreceptor input (Okada and Bunag, 1995). Both GABA\textsubscript{A} and GABA\textsubscript{B} receptors have an inhibitory influence on NTS activity involved in the baroreceptor inputs and chemoreceptor reflex in the rat (Ruggeri et al., 1996), cat (Miura et al., 1987) and rabbit (Suzuki et al., 1993). It has also been shown that GABA is a neurotransmitter regulating respiratory function in the brainstem (Murakoshi and Otsuka, 1985; Shao and Feldman, 1997).

In respect to gastrointestinal activity, distinguishable modulatory effects of GABA receptors have been reported in the gut (Ong and Kerr, 1983; Giotti et al., 1983; Krantis and Harding, 1987; Gentilini et al., 1992). The presence of GABA has been detected in the nodose ganglion, where neuronal cell bodies of centrally projecting vagal affereents are located (Bertilsson et al., 1976; Szabat et al., 1992). Electrophysiologically, GABA\textsubscript{A} depolarized vagal afferent neurons in the nodose ganglion projecting to the NTS (Wallis et al., 1982;
Ashworth-Preece et al., 1997). To date, however, whether GABA_A and GABA_B systems play a role in the medial NTS neurons that receive gastric vagal inputs has not been reported. In this study, we evaluated the GABAergic effects on the medial NTS neurons receiving gastric vagal inputs in the neonatal rat.

**Materials and Methods**

**Animal and surgical preparation.** The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Chicago. Experiments were performed on 29 Sprague-Dawley neonatal rats 1 to 3 days old. After the animal was deeply anesthetized with halothane, a craniotomy was performed and the forebrain was ablated at the caudal border of the pons by transection. The caudal brainstem and cervical spinal cord were isolated by dissection in modified Krebs' solution that contained (in mM) NaCl 128.0, KCl 3.0, NaH$_2$PO$_4$ 0.5, CaCl$_2$ 1.5, MgSO$_4$ 1.0, NaHCO$_3$ 21, mannitol 1.0, glucose 30.0 and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) 10.0. The stomach, connected to the esophagus, with the vagus nerves linking it to the brainstem, was kept, and all the other internal organs were removed. The preparation was then pinned with the dorsal surface up on a layer of Sylgard resin (Dow Corning) in a recording chamber. The preparation was superfused with Krebs' solution at 23 ± 1°C. The bathing solution was aerated continuously with a mixture of 95% O$_2$ and 5% CO$_2$ and adjusted to pH 7.35 to 7.45 (Murakoshi et al., 1985; Smith and Feldman, 1987; Barber et al., 1995; Yuan, 1996).

To investigate the distribution of the gastric GABA receptors that affect gastric vagally evoked NTS unitary activity, a whole-stomach preparation and a partial-stomach preparation were used (Yuan, 1996). The gastric mucosa structure of the proximal and the distal stomach under the dissecting scope appear distinctly different. This mucosa structure difference was used as a landmark to make the partial-stomach preparation. An incision was made at the greater curvature of the stomach body. The proximal stomach contained the fundus and the cardia. The distal stomach contained the pyloric antrum and the pylorus.

**Stimulation and recording methods.** A suction microelectrode was placed on the gastric vagal branch from the subdiaphragmatic vagi for electrical stimulation because only those neurons in the medial NTS receiving gastric vagal inputs were evaluated in this study. The gastric vagal fibers were stimulated with single or paired pulses of 200 μA for 0.2 msec at a frequency of 0.5 Hz by a Grass stimulator (model S8800) coupled to a stimulus isolation unit (SIU 5B, Grass Instruments, Quincy, MA). This current provided a stimulus intensity 1.5 to 2.0 times that required to produce maximal response intensity 1.5 to 2.0 times that required to produce maximal response.

Single tonic unitary discharges were recorded extracellularly in the medial NTS by glass microelectrodes filled with 2% pontamine sky blue in 0.5 M solution (Dow Corning) in a recording chamber. The recordings were performed with glass microelectrodes filled with 3 M NaCl, with an impedance of 10 to 20 MΩ (unitary discharge recordings; see Barber et al., 1995). A collision test was applied to identify ortho- and hetero-order NTS neurons in the gastric vagal afferent system were used in this study. For histological identification purposes, some glass microelectrodes were filled with 2% pontamine sky blue in 0.5 M medium acetate solution. After each unitary recording, current was applied at 5 μA in 5 sec on/10 sec off cycles for ~5 min, with the negative lead connected to the microelectrode.

**Experimental protocols.** GABA has both peripheral and central actions. To independently evaluate the gastric and brainstem effects of GABA on NTS neurons receiving gastric vagal inputs, a partition was made at the midthoracic level of the preparation. An agar seal separated the recording bath chamber into a brainstem compartment and a gastric compartment. GABA receptor agonists and antagonists were applied only to the gastric compartment or brainstem compartment and their effects on the NTS neuronal activity were evaluated. After each observation, drug or drugs were washed out from the compartment. The NTS neuronal responses observed during pretrial or pretreatment (control) were compared with post-trial (washout) to confirm that brainstem neuronal activity returned to the control level after washout. Tachyphylaxis was not evident in our experimental conditions because response to reapplication of a given concentration of agonist varied by <5%.

During the assessment of the distribution of gastric GABA receptors affecting NTS unitary activity, peripheral gastric effects of GABA receptor agonists were observed first in the whole-stomach preparation. Then, the proximal part or the distal part of the stomach was carefully removed, while unitary recording in the NTS was maintained. Approximately 5 min later, the effects of GABA agonists on the same NTS cells were again observed in the distal-stomach or proximal-stomach preparations.

In each experiment, the NTS unitary discharges were amplified with high-gain AC-coupled amplifiers (Axoprobe-1A, Axon Instruments, Burlingame, CA), displayed on a Hitachi digital storage oscilloscope (model VC-6525, Hitachi Denshi, Tokyo, Japan) and recorded on a Vetter PCM tape recorder (model 200, A.R. Vetter, Rebersburg, PA).

**Drugs.** The drugs used in this study, muscimol, baclofen, biccuculline and saclofen, were obtained from Research Biochemicals (Natick, MA).

**Data and statistical analysis.** The data from the NTS unitary activity were analyzed on the basis of action potential discharge rate and drug concentration-related effects. The number of action potentials in a given duration were measured under pretrial, trial and post-trial conditions. The control data (pretreatment) was normalized to 100%, and the NTS neuronal activities during and after trials were compared with the control data. Results are expressed as mean ± S.E. Data were analyzed using analysis of variance (ANOVA) for repeated measures and Mann-Whitney U test, with P < .05 considered statistically significant.

**Results**

Peripheral gastric effects of muscimol and baclofen on NTS unitary activity. Fifty-five tonic units receiving gastric vagal inputs were recorded in the medial NTS in the neonatal rat brainstem-gastric preparation. As shown in the sequential spike density histogram of figure 1, the discharge rate of a unit decreased after drug application. After application of the GABA_A receptor agonist muscimol and GABA_B receptor agonist baclofen to the gastric compartment, the firing rate of the NTS neurons was decreased. In 32 of 55 units observed, muscimol (30 μM; IC$_{50}$ = 2.0 μM) produced a concentration-dependent inhibitory effect of 36.2 ± 3.1% (mean ± S.E.) of the control level of the brainstem neuronal activity (fig. 2A). In 21 of 55 units observed, baclofen (30 μM; IC$_{50}$ = 1.5 μM) induced 31.0 ± 2.9% inhibition of the NTS unitary activity in a concentration-related fashion (fig. 2B). These 21 units also responded to muscimol (35.5 ± 3.3%). The differences in NTS neuronal discharge frequency between the control recording and the recording after muscimol (30 μM) and baclofen (30 μM) applications were significant (both P < .001). The remaining brainstem cells showed no response to these two GABA agonists. Some of these results are summarized in table 1.

Biccuculline (10 μM) and saclofen (10 μM), the GABA_A and GABA_B subreceptor antagonists, competitively antagonized these inhibitory effects by muscimol and baclofen, respectively (fig. 2, A and B). Biccuculline (10 μM) and saclofen (10 μM) did not have significant effects on the basal activity of NTS neurons.
Distribution of gastric GABA receptors affecting NTS unitary activity. Fifteen NTS units that responded to muscimol (30 μM) and 12 units that responded to baclofen (30 μM) were tested in both the whole-stomach and partial-stomach preparations. There were statistically significant differences in the gastric effects of muscimol (P < .01) and baclofen (P < .05) between the whole-stomach and proximal-stomach preparation in these inhibitory NTS neuronal responses. There also were statistical significant differences in the gastric effects of muscimol (P < .05) and baclofen (P < .05) between the whole-stomach and distal-stomach preparation. These results suggest that both the proximal and the distal stomach are important in the gastric effects of GABA agonists on gastric vagally evoked NTS unitary responses.

There was no statistically significant difference in the basal discharge rate of NTS neurons between the whole-stomach and partial-stomach preparations.

Central brainstem effects of muscimol and baclofen on NTS unitary activity. The central brainstem effects of muscimol and baclofen were evaluated on 51 gastric vagally evoked NTS unitary responses. Activity in NTS neurons was decreased in a concentration-related fashion after application of the GABA agonists, muscimol and baclofen, to the brainstem compartment. In 46 of 51 units observed, muscimol (30 μM; IC50 = 1.3 μM) produced an inhibitory effect of 54.1 ± 3.4% of the control level of the NTS neuronal activity (fig. 3A). In 40 of 51 units observed, baclofen (30 μM; IC50 = 1.1 μM) induced 48.9 ± 3.5% inhibition of the unitary activity (fig. 3B). For these 40 units, 38 units also responded to muscimol (54.9 ± 3.5%), and the two units responded only to baclofen (38.9%). The differences in NTS neuronal discharge frequency between the control recording and the recording after muscimol (30 μM) and baclofen (30 μM) applications were significant (both P < .001). Ordinate, discharge rate of NTS neurons expressed as percentage of control. The control activity level is normalized to 100%. Brackets indicate the mean ± S.E.M.

Bicuculline (10 μM) and saclofen (10 μM) competitively antagonized these inhibitory effects by muscimol and baclofen, respectively (fig. 3, A and B). Bicuculline (10 μM) and saclofen (10 μM) did not have significant effects on the basal activity of NTS neurons.

Discussion

The present study used an in vitro neonatal rat brainstem-gastric preparation to investigate the peripheral gastric and
central brainstem effects of GABA subreceptor agonists on NTS unitary activity. This preparation has the ability to change the local environment to ask questions concerning mechanisms of various synaptic and pharmacological events, while mimicking an in vivo preparation in which the gastric vagal inputs to the recorded NTS neurons can be identified. After the initial craniotomy under halothane anesthesia, the preparation eliminates the central effects of general anesthesia associated with in vivo studies. However, as neonatal animals were used in this study, circumspection should be exercised when extrapolating from the results obtained from an immature rat to an adult animal.

The NTS is the first central autonomic processing station, and a large number of cells responding to electrical stimulation of the gastric vagal branches have been recorded in the medial NTS in our previous experiments on the cat (Yuan and Barber, 1990). During these experiments, we often observed that within the same region, cardiovascular inputs could also be recorded in other NTS units, suggesting that several different visceral receptive inputs projected to different neurons in adjacent locations. In this study, we examined the peripheral gastric and central brainstem effects of GABAA and GABAB receptor agonists solely on NTS units processing gastric vagal inputs, an issue that has not been addressed in literature.

The GABAA and GABAB receptors have been localized in the gut in different species (Ong and Kerr, 1983; Giotti et al., 1983; Krantis and Harding, 1987; Nakajima et al., 1996). Ashworth-Preece et al. showed the presence of GABAA receptors located on the vagal afferent neurons of the rat (1997). Results from our study showed that peripheral gastric application of GABAA and GABAB receptor agonists decreased the discharge rate of NTS units in a concentration dependent manner. It seems that GABA agonists in the gastric compartment of our preparation interact with gastric GABA receptors, which in turn influence discharge frequency of the vagal afferent fibers.

It has been shown that GABA receptors exist in the gut myenteric plexus (Jessen et al., 1987) and muscular layers (Erdo et al., 1990). However, in our experimental paradigm, we were not able to differentiate whether the NTS neuronal responses to gastric GABA receptor agonist applications were mediated through the activation of GABA receptors directly on nerve fibers or were secondary to activation of receptors on gastric smooth muscle cells or both.

Results from a previous study, using the whole-stomach and the partial-stomach preparations, demonstrated that the distal stomach, not the proximal stomach, is important in the cholecystokinin gastric effects on brainstem neuronal activity (Yuan and Barber, 1993). This could be attributed to the fact that cholecystokinin has its binding sites located in the

### Table 1

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<tr>
<th></th>
<th>No. of NTS Units</th>
<th>Response to Muscimol</th>
<th>% of Response</th>
<th>Percent of Inhibition (mean ± S.E.)</th>
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<tr>
<td>Gastric effects</td>
<td>55</td>
<td>32</td>
<td>58.2</td>
<td>36.2 ± 3.1</td>
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<tr>
<td>Brainstem effects</td>
<td>51</td>
<td>46</td>
<td>90.2</td>
<td>54.1 ± 3.4</td>
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<tr>
<th></th>
<th>No. of NTS Units</th>
<th>Response to Baclofen</th>
<th>% of Response</th>
<th>Percent of Inhibition (mean ± S.E.)</th>
</tr>
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<tbody>
<tr>
<td>Gastric effects</td>
<td>55</td>
<td>21</td>
<td>38.2</td>
<td>31.0 ± 2.9</td>
</tr>
<tr>
<td>Brainstem effects</td>
<td>51</td>
<td>40</td>
<td>78.4</td>
<td>48.9 ± 3.5</td>
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Fig. 3. A, Central brainstem concentration-related effects of muscimol on NTS units. The effect of muscimol (●) in the presence of bicuculline (10 μM) has also been shown (■). B, Central brainstem concentration-related effects of baclofen on NTS units. The effect of baclofen (●) in the presence of saclofen (10 μM) has also been shown (■). The differences in unitary discharge frequency between the control recording and the recording after muscimol (30 μM) and baclofen (30 μM) were significant (both P < .001). Note different ordinate scale compared to that of figure 2.
pyloric sphincter, the antral circular muscle and the gastric mucosa at different stages of development (Robinson et al., 1987). In this study, we showed that both the proximal and the distal stomach are important in GABA effects on NTS neuronal activity. This result is consistent with the observation from an angiographic study, in which specific GABA\(_A\) binding sites were localized in both the body and antrum of the stomach (Erdo et al., 1990).

Neuronal cell bodies of centrally projecting vagal afferents are located in the nodose ganglion (Spyer, 1990) and the presence of GABA has been detected in the ganglion both chemically (Bertilsson et al., 1976) and immunocytochemically (Szabat et al., 1992). Functionally, GABA has been shown to depolarize rabbit nodose ganglion cells (Wallis et al., 1982). Broussard et al. demonstrated that esophageal premotor neurons within the NTS expressed GABA\(_A\) \(\alpha 1\) mRNA (Broussard et al., 1996). At the NTS, there is considerable evidence that GABA is a major inhibitory neurotransmitter. NTS is known to contain relatively high levels of GABA compared to other brain regions (Siemers et al., 1982). It is reasonable to assume that in our preparation GABA agonists in the gastric compartment act on gastric GABA receptors and, subsequently, cause the release of endogenous GABA at brainstem terminals which inhibit NTS neuronal activity.

Our results showed that the activity of medial NTS neurons receiving gastric vagal inputs was inhibited by both muscimol and baclofen application in the brainstem compartment. Interestingly, the percentage of NTS units that responded to GABA\(_A\) and GABA\(_B\) agonists is rather similar to the percentage of baroceptive NTS cells that responded to GABA\(_A\) and GABA\(_B\) agonists (Ruggeri et al., 1996). When comparing the percentage of NTS units responding to GABA application in the gastric compartment and in the brainstem compartment, we noticed that the latter initiated a significantly higher percentage of the NTS responses. In addition, the brainstem GABA exerted a higher magnitude of inhibition compared to the effects from the gastric GABA agonists. It has been shown that GABA\(_A\) agonist muscimol and GABA\(_B\) agonist PCPGABA may increase gastric acid secretion, acting through central cholinergic descending mechanisms (Yamasaki et al., 1971), whereas GABA\(_B\) agonist baclofen affected gastric acid secretion, acting via a central vagal pathway (Goto et al., 1985). Wang and Bieger (1991) observed that the injection of GABA\(_A\) antagonist bicuculline into the NTS resulted in repetitive esophageal contractions, and suggested that brainstem GABA neurons exert a powerful inhibition of deglutitive premotor elements governing pharyngeal and esophageal stages. Using a rat brainstem slice preparation, Brooks et al. (1992) demonstrated that inhibitory synaptic responses recorded in the NTS were blocked by bicuculline, and both presynaptic and postsynaptic responses to GABA\(_B\) receptor agonist baclofen were observed. In their experiment, NTS neurons were identified by electrical stimulation of the TS, and thus, the specific origin of inputs could not be identified. It seems that a certain number of NTS neurons recorded in their study were units receiving gastric vagal inputs observed in our experiment.

One of the axonal projections of the NTS neurons receiving GABAergic gastric vagal afferent inputs is the DMV, an area of the preganglionic parasympathetic motoneurons that provide vagal outflow to the viscera (Van Giersbergen et al., 1992). Synaptic connections between TS afferents and DMV have been demonstrated (Champagnat et al., 1986). DMV neurons are the major source of vagal motor fibers innervating the esophagus and stomach (Norman et al., 1985). Oikomoto et al. (1988) demonstrated that the delayed contraction of the stomach elicited by stimulation of the vagal trunk was inhibited by GABA acting on GABA\(_A\) receptors. Travagl et al. (1991) observed a spontaneous and evoked release of GABA acting at DMV motoneurons, and this inhibitory GABAergic input was mediated by GABA\(_B\) receptors. Evidence from pharmacological studies of DMV neurons that modulate gastrointestinal activity indicated that microinjection of GABA\(_A\) antagonist bicuculline into the rostral part of the DMV will increase gastric motility (Feng et al., 1990). Washabau et al. (1995) reported that the lower esophageal sphincter pressure, gastric motility and gastric acid secretion were influenced by GABA\(_B\) receptor mediated tonic inhibition of the vagal dorsal motor nucleus. Our results indicated a GABA inhibition in medial NTS neurons receiving gastric vagal afferents. It is plausible that an endogenous GABA release exerts an inhibitory control on premotor elements in DMV areas modulating gastric activity.

An analysis of the movements of the isolated stomach during distension showed the presence of intrinsic inhibitory and excitatory reflexes controlling a rich repertoire of gastric movements (Hennig et al., 1997). Execution of such complex motor activity may also require brainstem-mediated synaptic excitation and inhibition to ensure timing and coordination of different motoneuron pools. Further investigations are necessary to confirm this view and to show that GABA-mediated mechanisms do interact with excitatory responses in the control of gastric motility.

In summary, an in vitro neonatal rat brainstem-gastric preparation was used to evaluate the peripheral gastric and central brainstem effects of GABA\(_A\) and GABA\(_B\) subreceptor agonists on NTS neurons that responded to electrical stimulation of the gastric vagal fibers. Our results suggest that gastric and brainstem GABA\(_A\) and GABA\(_B\) receptors play an important role in modulation of brainstem neuronal activity and may play a role in regulating the digestive process.

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