A reevaluation of the effects of stimulation of the dorsal motor nucleus of the vagus on gastric motility in the rat

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Cruz, Maureen T., Erin C. Murphy, Niaz Sahibzada, Joseph G. Verbalis, and Richard A. Gillis. A reevaluation of the effects of stimulation of the dorsal motor nucleus of the vagus on gastric motility in the rat. Am J Physiol Regul Integr Comp Physiol 292: R291–R307, 2007. First published September 21, 2006; doi:10.1152/ajpregu.00863.2005.—Our primary purpose was to characterize vagal pathways controlling gastric motility by microinjecting l-glutamate into the dorsal motor nucleus of the vagus (DMV) in the rat. An intragastric balloon was used to monitor motility. In 39 out of 43 experiments, microinjection of l-glutamate into different areas of the DMV rostral to calamus scriptorius (CS) resulted in vagally mediated excitatory effects on motility. We observed little evidence for inhibitory effects, even with intravenous atropine or with activation of gastric muscle muscarinic receptors by intravenous bethanochol. Inhibition of nitric oxide synthase with Nω-nitro-l-arginine methyl ester (l-NAME) HCl did not augment DMV-evoked excitatory effects on gastric motility. Microinjection of l-glutamate into the DMV caudal to CS produced vagally mediated gastric inhibition that was resistant to l-NAME. l-Glutamate microinjected into the medial subnucleus of the tractus solitarius (mNTS) also produced vagally mediated inhibition of gastric motility. Motility responses evoked from the DMV were always blocked by ipsilateral vagotomy, while responses evoked from the mNTS required bilateral vagotomy to be blocked. Microinjection of oxytocin into the DMV inhibited gastric motility, but the effect was never blocked by ipsilateral vagotomy, suggesting that the effect may have been due to diffusion of oxytocin to the mNTS. Microinjection of substance P and N-methyl-D-aspartate into the DMV also produced inhibitory effects attributable to excitation of nearby mNTS neurons. Our results do not support previous studies suggesting that parallel excitation and inhibition pathways originating in the DMV caudal to CS. Our results do support previous findings of vagal inhibitory pathways originating in the DMV caudal to CS.

vagus nerve; nonadrenergic noncholinergic

THE DORSAL MOTOR NUCLEUS OF THE VAGUS (DMV) consists largely of vagal preganglionic neurons that project onto postganglionic neurons innervating the upper GI tract (11). Historically, it was believed that the vagus nerve provided only excitatory parasympathetic “drive” to the gut (2). However, recent reviews of vagal control of GI function propose that vagal efferents to the stomach consist of parallel excitatory and inhibitory pathways (2, 15, 47). One study on vagovagal reflex pathways in the rat indicates that excitatory and inhibitory DMV pathways have relatively equivalent roles in producing gastric relaxation (40). A more recent study suggests that activation of the inhibitory DMV pathway (as opposed to withdrawal of the excitatory DMV pathway) plays the major role in mediating reflex-induced fundus relaxation (13). Consistent with findings of earlier studies (20, 23, 45), nitric oxide was reported to be the chemical mediator of the inhibitory DMV pathway (13).

The excitatory pathway consists of preganglionic cholinergic neurons synapsing onto postganglionic cholinergic neurons. The inhibitory pathway has been proposed to be composed of two types of preganglionic vagal neurons, both of which originate from the DMV. The first type consists of a preganglionic cholinergic neuron synapsing onto a postganglionic nonadrenergic, noncholinergic (NANC) neuron (2, 15, 47). The second type is a preganglionic nitrergic neuron synapsing onto a postganglionic NANC neuron (20, 21). General acceptance of these pathways is evident in the range of published data obtained in the framework of parallel paths projecting to the stomach (1, 16, 18, 19, 39, 40, 49).

The preganglionic cholinergic DMV neurons that form the excitatory pathway to the stomach are found in the intermediate and rostral zones in the cat (33, 41) and in the intermediate zone in the rat (8, 44). (Note: The method for dividing the DMV into zones is described in MATERIALS AND METHODS). The preganglionic cholinergic DMV neurons that form the inhibitory pathway to the stomach were first considered to be exclusively in the caudal zone in the cat (41), but more recent data from the rat locate them also in the intermediate and rostral zones of the DMV (23, 39, 40). The preganglionic nitrergic neurons are described only in the rat and are reported to be in the caudal (20) and the rostral zones of the DMV (20, 21).

In our studies, we have noted the presence of functional parallel excitatory and inhibitory DMV vagal pathways to the lower esophageal sphincter (LES) and stomach in the cat (41). However, in the rat, we have not been able to obtain evidence of functional parallel excitatory and inhibitory DMV vagal pathways to the smooth muscle of the stomach. In our experiments, we have employed four different stimuli that engage the DMV and change gastric motility: esophageal distention, intravenous nicotine, intravenous CCK, and intravenous glucose. We demonstrated that each of these stimuli induced vagally mediated gastric inhibition (6, 7, 42). However, under these conditions, there was no evidence that activation of NANC postganglionic neurons was involved in the inhibition of gastric motility. Rather, the inhibition of motility was due to withdrawal of activity from an excitatory cholinergic DMV pathway. Likewise, Kobashi and colleagues (16) failed to obtain evidence of dual DMV pathways to the stomach of the rat. In their study, they showed that activation of water-responsive vagal afferents in the superior laryngeal nerve.

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produced gastric inhibition. Neurophysiological recordings revealed a decrease in the firing rate of DMV neurons in response to the administration of water into the larynx. This occurred in 10 caudal and 36 intermediate DMV neurons. It was inferred that inhibition of gastric motility was achieved by a reduction of activity in the excitatory DMV pathway. Consistent with this inference was the finding that intravenous atropine abolished the inhibition of gastric motility induced by administration of water into the larynx (17). No evidence for reflex-induced activation of an inhibitory DMV pathway was obtained.

The results of our previous studies and those of Kobashi and colleagues (16, 17) are incongruous with the existence of functional parallel excitatory and inhibitory DMV vagal pathways to the smooth muscle of the rat stomach. Thus, we set out to use the microinjection technique to reassess whether dual DMV pathways to the stomach exist and, if so, whether they are functionally important. Because most of the evidence for parallel inhibitory and excitatory vagal motor pathways to the stomach has been derived from data obtained by microinjection of drugs into the hindbrain of the rat (19, 21, 23, 29, 38, 40), we chose this species for our experimental model.

MATERIALS AND METHODS

Animals and Surgical Preparation

Experiments were performed on adult (250–350 g), male Sprague-Dawley rats (Taconic, MD) in accordance with the National Institutes of Health guidelines for the use of animals in research and with the approval of the Animal Care and Use Committee of Georgetown University, Washington, DC. Animals were fasted for 18–24 h, with water provided ad libitum. The anesthesia consisted of a mixture of urethane (800 mg/kg) and α-chloralose (60 mg/kg) dissolved in 3 ml of a 0.9% saline solution. Anesthesia was administered via intraperitoneal injection (3 ml/kg), with 0.2 ml supplements administered when necessary to maintain the animal unresponsive to toe pinch. Body temperature was maintained at 37°C with an infrared heating lamp. Animals were intubated to maintain a patent airway and to provide additional Institutes of Health guidelines for the use of animals in research and with the approval of the Animal Care and Use Committee of Georgetown University, Washington, DC. Animals were fasted for 18–24 h, with water provided ad libitum.

To monitor mean arterial blood pressure (i.e., 5 μV·V⁻¹·mmHg⁻¹), which, in turn, was connected to a bridge amplifier and data acquisition system (Powerlab; ADI Instruments, Colorado Springs, CO).

Microinjection Procedure and Histologic Verification of Microinjection Sites

Animals were placed in a prone position in an animal stereotaxic frame (Kopf Instruments, Tujunga, CA). Before stereotaxic surgery, all animals were pretreated with dexamethasone (0.8 mg sc) to minimize swelling of the brain. A partial craniotomy was performed to expose the dorsal medulla. The cerebellum was retracted, and the underlying dura and subarachnoid layers were cut and reflected.

In reevaluating the effects of DMV stimulation on gastric motility in the rat, we were guided by three overriding concerns. The first was the close proximity of the DMV to the nucleus tractus solitarius (NTS). These two hindbrain nuclei influencing gastric motility are separated by only 0.1 mm throughout most of their rostral-caudal extent (30), and this degree of separation narrows as one explores the DMV caudal to calamus scriptorius (CS) (35). The juxtaposition of these nuclei makes it difficult to selectively stimulate the DMV separately from the NTS. The second concern was to decrease the number of technical failures based on faulty placement of the micropipette into the dorsomedial medulla. In preliminary studies, we experienced a number of failed experiments when we based our micropipette placement solely on the stereotaxic coordinates of Paxinos and Watson (35). That is, histological examination of the brain would reveal micropipette placement that was inconsistent with the sites determined by the coordinates of Paxinos and Watson (35). Likely reasons for this were (1) the wide range in body weights (250–350 g) and presumably corresponding variations in brain size of the rats provided to us, and (2) the aforementioned juxtaposition of the DMV and NTS. We reduced the number of failed experiments when, in addition to using stereotaxic criteria, we used microinjection of l-glutamate as a pharmacological tool to locate specific hindbrain nuclei.

It should be noted that this second concern has been a factor in the microinjection protocol used by other investigators to functionally locate the DMV before microinjection of test agents such as substance P and orexins (18, 19). In these studies, substance P and orexins were found to activate DMV NANC neurons. More importantly, precise positioning of the micropipette tip was based on observing a “brief but marked increase in IGP and antral motility” upon microinjection of l-glutamate (19). Once placement of the pipette tip was ensured by the expected gastric motor responses, microinjections of substance P [and NKR-selective agents] were made (19). In that study, microinjection of substance P into the DMV was reported to evoke gastric motor inhibition (19).

The third concern that dictated our micropipette placement was the inclusion of specific regions of the DMV that have been proposed as origins of NANC DMV pathways to the stomach. One region is an area of preganglionic cholinergic neurons in the rostral lateral portion of the DMV (39), and the other region is an area of NO synthase-containing preganglionic neurons in the rostral DMV (20, 21).

In the majority of our studies, we placed the micropipette tip in the DMV or NTS using the coordinates of Paxinos and Watson (35). We then microinjected 30 nl of a 16.7 mM solution of l-glutamate to precisely locate the DMV and NTS. For all of the studies, the CS (the caudal tip of the AP) was used as a reference point for calculating the coordinates for micropipette placement. Paralleling the scheme used by Loewy to divide the NTS into three different areas (26), we divided the DMV into three zones. This division is based on the position of the DMV relative to the area postrema (AP) and consists of a caudal zone (area of the DMV...
behind CS), an intermediate zone (area of the DMV that runs parallel to the AP), and a rostral zone (area of the DMV that lies anterior to the AP). Stereotaxic coordinates for microinjection into the rostral DMV were 0.7 to 1.1 mm rostral to CS (AP was not present at this level), 0.4 to 0.8 mm lateral to midline, and 0.4 to 0.7 mm below the dorsal surface of the medulla. Coordinates for the intermediate DMV were 0.1 to 0.6 mm rostral to CS, 0.3 to 0.6 mm lateral from the midline, and 0.5 to 0.9 mm below the dorsal surface of the medulla. As mentioned above, the precise location of the rostral and intermediate DMV was assessed by microinjecting 1-glutamate into the stereotaxically designated site and noting an increase in IGP (8). Coordinates for the caudal DMV were 0.2 to 0.7 mm caudal to CS, 0.2 to 0.4 mm lateral from the midline, and 0.7 to 1.0 mm below the dorsal surface of the medulla. Precise location of the caudal DMV was assessed by microinjecting 1-glutamate into the stereotaxically designated site and noting a decrease in IGP without a significant decrease in arterial blood pressure (41). Coordinates for the mNTS were 0.3 to 0.5 mm rostral to CS, 0.4 to 0.8 mm lateral to midline, and 0.1 to 0.6 mm from the dorsal surface of the medulla. Precise location of the mNTS was assessed by microinjecting 1-glutamate into the stereotaxically designated site and noting decreases in both IGP and arterial blood pressure (8, 44). Coordinates for the commissural NTS were 0.2 to 0.7 mm caudal to CS, 0.1 to 0.6 mm lateral from the midline, and 0.6 to 0.8 mm from the dorsal surface of the medulla. In studying the commissural NTS, we used only stereotaxic coordinates to guide us in micropipette placement since, to our knowledge, there are no reports of the gastric motor effects of commissural NTS stimulation by 1-glutamate.

If 1-glutamate microinjection did not produce the characteristic responses evoked from the rostral, intermediate, or caudal DMV, or from the mNTS, we moved the micropipette tip in 100-μm increments to vertical, rostral-caudal, and medial-lateral sites until the characteristic response was obtained.

To study the area of preganglionic cholinergic neurons in the rostral-lateral portion of the DMV (an area described as containing NANC inhibitory neurons) (39), we microinjected 1-glutamate into an area 0.4 to 0.9 mm rostral to CS (encompassing part of the intermediate DMV), 0.6 to 1.0 mm lateral to midline, and 0.7 mm below the dorsal surface of the medulla (see Fig. 11G of Ref. 39).

All microinjections in the brain stem were made with double-barreled glass micropipettes (inner diameter: 0.3 mm; tip diameter: 30–60 μm; Frederick Haer, Bowdoinham, ME) angled at 30° from the perpendicular. These micropipettes were attached to PE-90 tubing. The tubing was fastened to a syringe, and drugs were loaded and ejected using negative or positive pressure, respectively. Drugs were injected in a 30-nl volume, as monitored by calibration tape affixed to the pipette. Injections were administered within 5–10 s by hand-controlled pressure.

At the end of each experiment, the animal was euthanized with an overdose of pentobarbital. The brain was removed and fixed in a mixture of 4% paraformaldehyde and 20% sucrose for at least 24 h. It was then cut into 50-μm-thick coronal serial sections and stained with neutral red. The location of microinjection sites was studied in relation to nuclear groups using the atlas of Paxinos and Watson (35). Camera lucida drawings were completed for each experiment to document all microinjection sites.1

**Experimental Protocols**

**General protocol.** In all experiments, a stable baseline IGP and BP recording was obtained for at least 10 min before any experimental manipulations were initiated. The micropipette was inserted unilaterally, and the animal was allowed to stabilize for at least 2 min. Thereafter, 1-glutamate (30 nl of a 16.7 mM solution; 500 pmol total) was used as a pharmacological tool to identify pipette location (see Microinjection Procedure and Histologic Verification of Microinjection Sites). A minimum of 10 min elapsed between 1-glutamate injections. This time interval was observed to allow full recovery and obtain reproducible responses. When drugs other than 1-glutamate were studied, the time interval between injections had to be determined for each agent. After establishing the effect of a microinjected drug (e.g., 1-glutamate), vagotomy, and/or systemic administration of other drugs (e.g., 1-NNAME) were used before repeat microinjection of the drug of interest.

**Protocol for studying the effect of 1-NNAME on 1-glutamate responses.** In this study, at least two reproducible responses to microinjection of 16.7 mM 1-glutamate were obtained. Next, 1-NNAME (10 mg/kg iv) was administered to inhibit nitric oxide synthase (NOS) and abolish NANC effects on gastric motility. This dose of 1-NNAME has been shown to block NANC effects on gastric motility in the rat (13, 19, 23, 45, 46). Once gastric motility and arterial blood pressure stabilized (usually 10–15 min after 1-NNAME administration), 1-glutamate microinjection was repeated. Next, ipsilateral vagotomy and/or bilateral vagotomy was performed, and the 1-glutamate microinjection was repeated.

**Protocol for studying the effect of atropine methyl bromide on 1-glutamate responses.** In this study, a protocol similar to the one described for 1-NNAME experiments was used. Instead of 1-NNAME, atropine methyl bromide (a permanently charged molecule that does not cross the blood-brain barrier) was administered to block peripheral muscarinic receptors. The dose used in our experiments (0.1 mg/kg iv) is within the dose range of atropine that has been shown to completely block the muscarinic receptors in the periphery (9).

**Protocol for studying the effect of 1-glutamate during infusion of bethanechol.** On the basis of the studies of Lewis et al. (23), these experiments assessed the effect of DMV excitation (by 1-glutamate) on gastric motility during maximal stimulation of gastric muscarinic receptors. First, 1-glutamate was microinjected to locate the DMV. Next, bethanechol (30 μg·kg−1·min−1), a peripheral muscarinic receptor agonist, was infused continuously via the femoral vein to excite the gastric smooth muscle muscarinic receptors. Once a robust, stable bethanechol response was established, microinjection of 1-glutamate into the DMV was repeated (while the bethanechol infusion continued). If 1-glutamate produced an effect during the continuous bethanechol infusion, then microinjection was repeated to ensure a reproducible response. Next, ipsilateral and bilateral vagotomies were performed in sequence, with 1-glutamate microinjections between the sectioning of each nerve. If 1-glutamate had no effect during the bethanechol infusion (1-glutamate was tested at least twice), the infusion was terminated. The animal was allowed to recover from the effects of bethanechol infusion (60–90 min was required), then was retested with 1-glutamate microinjected into the DMV before and after ipsilateral vagotomy.

**Protocols for replicating key experiments from previous reports.** After 1-glutamate was used to identify the hindbrain nucleus of interest, we studied the effects of substance P, oxytocin, CRF, and N-methyl-D-aspartate (NMDA) on gastric motility. CRF was also tested under conditions of continuous bethanechol infusion. The same protocol used to test unilateral microinjection of 1-glutamate was used to test the three neuropeptides and NMDA. Additional information about these protocols is provided in the RESULTS section.

**Data Analysis**

Data were analyzed using Chart Software (ADI Instruments). Figure 1 illustrates the end points and values used for analyzing gastric motility. Baseline gastric tone is defined by the value (in

1 Although the microinjection sites for all studies were documented, some are not presented because of space limitations. This information is available on request.
mmHg) represented by A, and the value (in mmHg) of the peak amplitude of baseline contractions is represented by B. Changes in gastric tone and motility in response to microinjection of a drug were compared with a 3-min baseline recording. The 3 min of baseline were divided into 1-min samples, averaged, and compared with the effect of the drug.

In analyzing the effect of experimental procedures on gastric motility, we used different approaches, depending on whether the procedure increased motility (i.e., effects evoked from the intermediate and rostral zones of the DMV) or decreased motility (i.e., effects evoked from the NTS and caudal zone of the DMV). For analyzing increases in motility, two end points were studied. The first was the peak increase in IGP, which was considered as an indicator of the strength of the maximum contraction produced. This value (produced by drug Y) was equal to the difference (in mmHg) between the value of B and C in Fig. 1. The second end point was the area under the curve (AUC) of the motility response that served as an indicator of the total change in gastric motility. This value (produced by drug Y and represented by the shaded area above the line extending horizontally from A) was compared with the AUC of the baseline sample. For analyzing decreases in motility, the end points of interest were gastric tone and AUC of the motility response. In Fig. 1, decreases in gastric tone (as produced by drug X) were taken as the difference between A and D, and AUC of the motility response was defined as the summed area over time (produced by drug X and represented by the shaded area below the line extending horizontally from A). For obtaining AUC values for experimental interventions that decreased motility, we used only the value for the summed area produced by the intervention (see Fig. 1).

Changes in mean BP were also analyzed. The mean BP over a 3-min baseline period was compared with the mean BP following microinjection of drug. In cases in which microinjection of a drug caused a drop in mean BP, the nadir of the response was measured and compared with baseline. Similarly, in cases in which BP increased, the peak rise was measured and compared with baseline.

For experiments in which a drug was injected twice (under identical experimental conditions), the two responses were averaged to determine a mean response. Data are presented as means ± SE. A paired t-test was performed when animals served as their own controls. An unpaired t-test (independent samples) was performed on data from separate control and experimental groups. A one-sample t-test was performed to determine whether a group mean was significant from zero. In all cases, $P < 0.05$ was the criterion used to denote statistical significance.

Drugs

Drugs used for the studies were as follows: urethane, α-chloralose, dexamethasone sodium phosphate, L-glutamate, L-NAME, bethanecol HCl, atropine methyl bromide, sodium nitroprusside (SNP), hexamethonium bromide, substance P, NMDA, CRF (human/rat), and oxytocin. All drugs were purchased from Sigma-Aldrich (St. Louis, MO), with the exceptions of dexamethasone, which was purchased from American Regent Laboratories (Shirley, NY), substance P from Bachem (Torrance, CA), and oxytocin from Phoenix Pharmaceuticals (Belmont, CA). All drugs were dissolved in 0.9% saline, and the pH of drug solutions used in microinjection studies was brought to 7.0–7.4. The only exception was CRF, which was kept at pH 3.5 because the drug precipitated at physiological pH. To control for this, normal saline with pH 3.5 was tested and found to have no effects on gastric motility and mean blood pressure (data not shown).

The pivotal drug used in our study was L-glutamate. On the basis of a dose-response curve derived from microinjection studies in the rat DMV (4), 30 nl of a 16.7 mM solution of L-glutamate (i.e., 500 pmol) was selected for study. Using a 60-nl injectate volume, this earlier study tested L-glutamate solutions ranging from 0.5 mM to 50 mM, and concentration-dependent increases in gastric motility were observed upon microinjection of this amino acid into the DMV. The half-maximal effective dose (ED50) for the response was 2.67 mM (4). Hence, 30 nl of a 16.7 mM solution was estimated to be near the top of the dose-response curve but was not on the plateau phase of the curve. Most importantly, this dose was lower than the dose range found to exert depolarization blockade of neurons (25). Finally, the 16.7 mM solution is in the same range used by two other reports that have studied the effects of exciting CNS neurons on autonomic outflow to the periphery (3, 44).

**RESULTS**

**Studies Distinguishing DMV Effects from NTS Effects on Gastric Motility**

Most microinjection studies of the DMV either focus on the intermediate zone or include this portion of the DMV in their investigation (19, 21, 23, 29, 36, 38, 40). At this level, the mNTS is the subnucleus of the NTS that lies directly dorsal to the DMV (26, 34). Thus the starting point of our investigation was to compare the changes in gastric motility evoked by microinjection of L-glutamate at the intermediate zone of the DMV with those evoked from the mNTS.

**L-Glutamate microinjection into the intermediate DMV.** For these DMV studies, we focused on two facets of gastric motility as end points (see MATERIALS AND METHODS). Data obtained from eight animals are summarized in Table 1. Unilateral microinjection of 30 nl of 16.7 mM L-glutamate (i.e., 500 pmol) produced a 4.5 ± 0.7 mmHg increase in IGP. Similarly, L-glutamate microinjection produced a 104.6 ± 19.1 mmHg · s increase in the AUC. These increases in gastric motility occurred without any significant change in BP. A representative experiment showing L-glutamate-induced increases in IGP and AUC appears as Fig. 2A. The onset of effect was within 8.6 s, peak increase occurred at 23.3 s, and the duration of the effect was 63.2 s. This time action curve of the excitatory effect of L-glutamate on gastric motility was typical
of all eight experiments. The excitatory effect of L-glutamate could be reproduced if a 10-min interval was used between microinjections (Fig. 2A). The location of the microinjection pipette tip in the intermediate DMV is shown in Fig. 2B. A summary of all microinjection sites for this series of experiments appears as Fig. 3. In the eight animals studied, successful microinjection of L-glutamate into the intermediate DMV always evoked an increase in gastric motility. In no instance was a decrease in motility observed.

Two types of control experiments were performed. In the first, vehicle for L-glutamate (i.e., 30 nl of saline) was microinjected unilaterally into the intermediate DMV. Four experiments were performed, and the change in peak IGP was +0.2 ± 0.2 mmHg, while the change in AUC was +10.5 ± 12.2 mmHg·s. These values were not statistically significant. In the second set of control experiments, unilateral microinjections of 16.7 mM L-glutamate were given just outside both the DMV and the subnuclei of the NTS. Four experiments were performed, and the change in peak IGP was −0.1 ± 0.1 mmHg and the change in AUC was −1.2 ± 0.9 mmHg·s. These values were also not statistically significant. The microinjection sites for the control experiments appear in Fig. 3.

Next, we studied the effect of ipsilateral cervical vagotomy on L-glutamate-induced increases in IGP and AUC. This investigation was performed in the same eight animals described previously. In these experiments, L-glutamate was microinjected unilaterally into the intermediate DMV. This was done at least twice to ensure reproducible responses. Next, the cervical vagus nerve ipsilateral to the L-glutamate microinjection site was sectioned. Microinjection of L-glutamate was repeated, and the results obtained are summarized in Table 1. As can be noted, ipsilateral vagotomy abolished the excitatory effect of L-glutamate on gastric motility. A representative experiment illustrating the effect of ipsilateral vagotomy is shown in Fig. 2A.

### Table 1. Effects of vagotomy, L-NAME, bethanecol, and atropine on changes in IGP, AUC, and BP produced by unilateral microinjection of 500 pmol of L-glutamate into either the DMV or the NTS

<table>
<thead>
<tr>
<th>Hindbrain Site Studied</th>
<th>Treatment</th>
<th>n</th>
<th>Control Response to L-Glutamate</th>
<th>AUC, mmHg·s</th>
<th>Mean BP, mmHg</th>
<th>Control Response to L-Glutamate</th>
<th>AUC, mmHg·s</th>
<th>Mean BP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate DMV</td>
<td>Ipsilateral vagotomy</td>
<td>8</td>
<td>+4.5 ± 0.7†</td>
<td>+104.6 ± 19.1†</td>
<td>+0.8 ± 0.7</td>
<td>+1.3 ± 0.3</td>
<td>+13.6 ± 6.3*</td>
<td>+19.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>L-NAME</td>
<td>4</td>
<td>+2.9 ± 1.2</td>
<td>+49.7 ± 27.5</td>
<td>−0.6 ± 3.6</td>
<td>+3.0 ± 1.4</td>
<td>+43.3 ± 16.3</td>
<td>−12.6 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>Bethanecol</td>
<td>6</td>
<td>+2.3 ± 0.5†</td>
<td>+48.5 ± 8.4†</td>
<td>+0.6 ± 2.2</td>
<td>+1.4 ± 0.8</td>
<td>+42.5 ± 15.7†</td>
<td>+0.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>6</td>
<td>+3.8 ± 1.2</td>
<td>+60.8 ± 16.3†</td>
<td>−5.4 ± 4.7</td>
<td>−0.1 ± 0.1</td>
<td>+3.2 ± 1.5</td>
<td>−9.5 ± 5.0</td>
</tr>
<tr>
<td>Medial NTS</td>
<td>Ipsilateral vagotomy</td>
<td>6</td>
<td>−1.0 ± 0.2†</td>
<td>−47.3 ± 8.0†</td>
<td>−18.1 ± 2.2†</td>
<td>−0.8 ± 0.2†</td>
<td>−43.3 ± 9.2†</td>
<td>−16.1 ± 1.7†</td>
</tr>
<tr>
<td></td>
<td>Bilateral vagotomy</td>
<td>6</td>
<td>−1.0 ± 0.2†</td>
<td>−47.3 ± 8.0†</td>
<td>−18.1 ± 2.2†</td>
<td>0.0 ± 0.1†</td>
<td>−2.0 ± 0.6†</td>
<td>−20.2 ± 5.2†</td>
</tr>
<tr>
<td></td>
<td>L-NAME</td>
<td>5</td>
<td>−1.5 ± 0.3†</td>
<td>−88.9 ± 55.8</td>
<td>−19.4 ± 2.1†</td>
<td>−0.7 ± 0.2†</td>
<td>−38.2 ± 10.8†</td>
<td>−21.9 ± 2.9†</td>
</tr>
<tr>
<td>Caudal DMV</td>
<td>Ipsilateral vagotomy</td>
<td>5</td>
<td>−0.7 ± 0.1†</td>
<td>−32.3 ± 5.2†</td>
<td>+0.8 ± 2.7†</td>
<td>−0.0 ± 0.0†</td>
<td>−0.1 ± 1.9†</td>
<td>+4.2 ± 2.8</td>
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<tr>
<td></td>
<td>L-NAME</td>
<td>3</td>
<td>−1.1 ± 0.4</td>
<td>−77.7 ± 17.6†</td>
<td>+2.9 ± 3.3</td>
<td>−0.9 ± 0.3</td>
<td>−35.2 ± 3.2†</td>
<td>+4.6 ± 3.2</td>
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<tr>
<td>Commissural NTS</td>
<td>Ipsilateral vagotomy</td>
<td>4</td>
<td>−0.9 ± 0.4</td>
<td>−53.1 ± 22.9</td>
<td>+22.8 ± 4.2†</td>
<td>−1.0 ± 0.4</td>
<td>−77.6 ± 28.0</td>
<td>−7.2 ± 12.9</td>
</tr>
<tr>
<td></td>
<td>Bilateral vagotomy</td>
<td>4</td>
<td>−0.9 ± 0.4</td>
<td>−53.1 ± 22.9</td>
<td>+22.8 ± 4.2†</td>
<td>−0.2 ± 0.2†</td>
<td>−13.7 ± 8.0</td>
<td>−1.4 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>L-NAME</td>
<td>4</td>
<td>−0.6 ± 0.1†</td>
<td>−32.7 ± 13.9</td>
<td>+23.3 ± 7.5</td>
<td>−0.9 ± 0.4</td>
<td>−29.2 ± 18.8</td>
<td>−9.3 ± 28.3</td>
</tr>
</tbody>
</table>

Values presented are means ± SE. IGP, intragastric pressure; AUC, area under the curve; BP, mean arterial blood pressure; DMV, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; L-NAME, N°-nitro-L-arginine methyl ester. *P < 0.05, paired t-test; †P < 0.05, one-sample t-test.
in gastric tone and a $-32.3 \pm 5.2$ mmHg $\cdot$ s decrease in AUC. There was also a small increase in BP. Ipsilateral vagotomy abolished the decreases in gastric motility (Table 1). A representative experiment showing L-glutamate-induced decreases in gastric motility appears as Fig. 5A. The onset of effect was within 2.9 s, peak decrease occurred at 15.5 s, and the duration of effect was 65.0 s. This time action curve of the inhibitory effect of L-glutamate on gastric motility was typical of all five experiments. The inhibitory effect of L-glutamate could be reproduced if microinjections were done at 10-min intervals. The location of the microinjection pipette tip in the caudal DMV is shown in Fig. 5B. In one control animal, saline was microinjected into the caudal DMV and was found to have no effect on IGP. A summary of all microinjection sites for this series of experiments can be seen in Fig. 3.

**L-glutamate microinjection into the commissural NTS.** In the caudal brain stem, the region of the NTS that abuts the caudal DMV is the commissural NTS (comNTS) (26, 34). For these studies, the relevant end points were gastric tone and AUC. Data obtained from four animals are summarized in Table 1. L-glutamate microinjected unilaterally into the comNTS tended to decrease gastric tone as noted by the reductions in IGP and AUC. In addition, L-glutamate microinjected into the comNTS increased the mean BP. Ipsilateral vagotomy was tested on the L-glutamate-induced responses and was found to have no effect on the L-glutamate-induced changes in gastric motility. Bilateral cervical vagotomy, however, did counteract the change in IGP evoked by microinjection of L-glutamate. Both ipsilateral and bilateral cervical vagotomy counteracted the increases in BP (Table 1). A sample recording of one of the experiments is depicted in Fig. 6A, and the location of the microinjection pipette tip in the comNTS is shown in Fig. 6B. In two animals, saline was microinjected into the comNTS and was found to have little effect on IGP. A summary of all the microinjection sites is shown in Fig. 3.

**Studies of Specific DMV Regions Purported to Contain NANC Neurons**

The preganglionic DMV neurons that form the inhibitory pathway to the stomach have generally been considered to be exclusively in the caudal zone (2, 17, 41), but data from three studies performed in the rat locate them also in two specific areas of the DMV rostral to CS. Preganglionic cholinergic neurons in the rostrolateral DMV have been described as NANC inhibitory neurons (39), and nitrergic neurons in the rostral DMV have been suggested to function as another inhibitory DMV pathway (20, 21). The purpose of this series of experiments was to investigate the effects that stimulation of these two areas of the DMV would have on gastric motility.

**L-glutamate microinjection into the rostrolateral portion of the DMV.** To target this site, we microinjected L-glutamate 0.4 to 0.9 mm rostral to CS (encompassing both the intermediate and rostral DMV), 0.6 to 1.0 mm lateral to midline, and 0.7 mm from the dorsal surface of the medulla, a location more lateral than our intermediate DMV studies (see RESULTS, L-glutamate microinjection into the intermediate DMV). As described in the MATERIALS AND METHODS, L-glutamate-induced IGP response was not used to position the micropipette tip.

Ten rats were studied. In one animal, both DMVs were studied. Hence, the total number of experiments in this series of studies was 11. Of the 11 experiments, three exhibited an increase in IGP when L-glutamate was microinjected into the rostral lateral DMV. The IGP increased by $+3.6 \pm 1.1$ mmHg. AUC also changed by $+76.1 \pm 37.3$ mmHg $\cdot$ s. No change in mean BP was observed ($+0.9 \pm 3.5$ mmHg). In two of these animals, ipsilateral vagotomy prevented the increase in IGP.

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**Fig. 2. Response to unilateral microinjection of L-glutamate into the dorsal motor nucleus of the vagus (DMV) of the rat. A:** experimental tracing of intragastric pressure (IGP) and blood pressure (BP). The first two responses are control responses to L-glutamate. Following ipsilateral vagotomy (Ipsi Vx), repeat microinjection of L-glu did not increase IGP. **B:** photomicrograph of the DMV microinjection site (indicated by arrow). DMV, dorsal motor nucleus of the vagus; NTS, nucleus tractus solitarius; XII, hypoglossal nucleus; AP, area postrema; and CC, central canal.
In the third animal, atropine methyl bromide (iv) countered the increase in IGP. After ipsilateral vagotomy or atropine treatment, the increase in IGP was $+0.2 \pm 0.1$ mmHg.

In five experiments, L-glutamate microinjection decreased IGP ($-0.4 \pm 0.1$ mmHg, $P < 0.05$) but had no significant effect on AUC ($-25.1 \pm 13.5$ mmHg $\cdot$ s, $P > 0.05$). In four experiments, ipsilateral vagotomy did not block the decrease in IGP. In three of these animals, the remaining vagus nerve was sectioned, and consequently, the decrease in IGP was blocked. In the fifth animal, atropine methyl bromide (iv) prevented the decrease in IGP. After bilateral vagotomy ($n = 3$) or atropine treatment ($n = 1$), the L-glutamate-induced decrease in IGP was $-0.1 \pm 0.1$ mmHg. These data suggest that the decrease in IGP observed with L-glutamate

![Fig. 3. Camera lucida reconstruction detailing microinjection sites of L-glutamate (30 nl of a 16.7 mM solution) and saline into the rostral, intermediate, and caudal zones of the DMV, the mNTS, and the comNTS. Coordinates listed describe locations relative to calamus scriptorius.](image-url)
L-glutamate microinjected into an area of NOS-containing preganglionic neurons in the rostral DMV. L-glutamate (30 nl of a 16.7 mM solution) was used to precisely localize the micropipette tip in the rostral DMV using the stereotaxic coordinates of Krowicki and colleagues (19, 21). In the study that we were attempting to confirm (19), the investigators also functionally located the rostral DMV with L-glutamate before microinjection of test agents. In five animals, unilateral microinjection of L-glutamate into the rostral DMV produced a +5.1 ± 0.8 mmHg (P < 0.05) increase in IGP and an +87.5 ± 12.0 mmHg·s increase in AUC. In the same five animals, ipsilateral vagotomy was performed, and repeat administration of L-glutamate into the rostral DMV had no significant effect on either IGP (-0.4 ± 0.2 mmHg) or AUC (-6.3 ± 3.5 mmHg·s). Histological examination verified that the micropi-

microinjected into the rostralateral portion of the DMV was due to excitation of NTS neurons.

In one experiment, L-glutamate microinjected into the rostralateral DMV resulted in a decrease in IGP that was blocked by ipsilateral vagotomy. In the remaining two experiments in this series, the L-glutamate evoked no effect on gastric motility in one animal, and the microinjection site was too medial in the other animal. A summary of all microinjection sites for this group of experiments appears in Fig. 3.

Thus, of 11 experiments performed where L-glutamate was microinjected into the rostralateral portion of the DMV, only one experiment provided evidence for the existence of an inhibitory DMV pathway to the stomach.
Studies Aimed at Unmasking a NANC Pathway From the Intermediate DMV to the Stomach

Three studies were carried out to unmask a NANC gastric pathway originating from the intermediate zone of the DMV. Each study employed a different strategy to reveal a NANC pathway to the stomach. In the first study, the NOS inhibitor L-NAME was used to assess whether loss of the mediator of a NANC pathway originating from the intermediate zone of the DMV. Microinjection sites for the L-NAME studies were all located in the intermediate DMV (data not shown). In addition, we assessed whether L-NAME pretreatment would counteract the decreases in gastric motility evoked by microinjecting L-glutamate into either the mNTS or the caudal zone of the DMV. The mNTS data indicate that, although there was a tendency for L-NAME pretreatment to reduce the effect of L-glutamate, there was no statistically significant effect on the L-glutamate-induced decreases in gastric motility (Table 1). Because of the extremely close proximity of the DMV to the NTS at the caudal level of the DMV, only three animals could be tested with L-NAME. The data indicate that L-NAME pretreatment does not counteract L-glutamate-induced decreases in gastric motility (Table 1).

The second strategy to reveal a NANC pathway was to produce gastric muscarinic receptor stimulation by acute intravenous infusion of bethanechol. Other investigators have employed this technique to unmask a NANC vagal pathway to the stomach (23). Using this approach, we would expect that microinjection of L-glutamate following bethanechol infusion would excite the pool of DMV neurons in the intermediate DMV that synapse with NANC neurons innervating the stomach. If this pool of DMV neurons were present, a decrease in gastric motility should be apparent. The detailed protocol used for this study is described in the MATERIALS AND METHODS section. Studies were carried out in nine animals. In six of the nine experiments, L-glutamate microinjected during bethanechol infusion had no significant effect on IGP but did significantly increase AUC (Table 1). A representative experiment is shown in Fig. 7.

In the three remaining experiments, L-glutamate microinjection produced its usual increase in gastric motility (IGP increased by +3.8 ± 0.7 mmHg) before bethanechol treatment. However, during bethanechol infusion, L-glutamate microinjection decreased IGP (−2.3 ± 0.3 mmHg, P < 0.05). Subsequent ipsilateral vagotony abolished the decrease in IGP (+0.4 ± 0.1 mmHg after ipsilateral vagotomy). The AUC was +55.9 ± 8.7 mmHg·s (P < 0.05) before bethanechol and did not change significantly after bethanechol administration. A summary of all the microinjection sites for this series of experiments is shown in Fig. 8. It can be noted in Fig. 8 that for four of the six rats in which L-glutamate increased IGP, the pipette tip was at least +0.4 mm or more rostral to the CS. In the three rats in which L-glutamate decreased IGP, the pipette tip was only +0.2 to +0.3 mm rostral to CS. Hence, it is possible that L-glutamate may have diffused to the caudal DMV in these three rats.

In the third study, we assessed whether L-glutamate microinjected into the intermediate DMV would evoke gastric inhibition after blocking the excitatory cholinergic DMV pathway with systemically administered atropine methyl bromide (0.1 mg/kg iv). Four experiments were performed. As can be noted in Table 1, prior to treatment with atropine methyl bromide, L-glutamate microinjected into the DMV increased IGP and AUC by +3.8 ± 1.2 mmHg and +60.8 ± 16.3 mmHg·s, respectively. After treatment with atropine methyl bromide, no significant changes in these indices of gastric motility were noted. Microinjection sites for the atropine methyl bromide studies were all localized in the intermediate DMV (data not

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**Table 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>L-glutamate (mmHg)</th>
<th>AUC (mmHg·s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.8 ± 0.7</td>
<td>55.9 ± 8.7</td>
</tr>
<tr>
<td>2</td>
<td>2.3 ± 0.3</td>
<td>50.3 ± 8.2</td>
</tr>
<tr>
<td>3</td>
<td>0.4 ± 0.1</td>
<td>45.2 ± 8.1</td>
</tr>
<tr>
<td>4</td>
<td>3.8 ± 1.2</td>
<td>60.8 ± 16.3</td>
</tr>
<tr>
<td>5</td>
<td>60.8 ± 16.3</td>
<td>65.9 ± 18.2</td>
</tr>
<tr>
<td>6</td>
<td>0.2 ± 0.3</td>
<td>30.4 ± 7.2</td>
</tr>
<tr>
<td>7</td>
<td>0.3 ± 0.4</td>
<td>35.7 ± 8.3</td>
</tr>
<tr>
<td>8</td>
<td>0.4 ± 0.1</td>
<td>40.8 ± 8.8</td>
</tr>
<tr>
<td>9</td>
<td>0.5 ± 0.2</td>
<td>45.9 ± 9.1</td>
</tr>
</tbody>
</table>
Fig. 7. Response to unilateral microinjection of 30 nl of 16.7 mM L-glutamate into the DMV of the rat before and during continuous infusion of bethanechol (30 μg/kg/min). Note the drop in BP and increase in gastric tone and phasic contractions caused by bethanechol. L-glutamate had no effect on gastric motility during bethanechol infusion.

Fig. 8. Camera lucida reconstruction detailing sites of L-glutamate (16.7 mM solution) microinjected into the intermediate zone of the DMV during bethanechol infusion (n = 9). Coordinates listed describe locations relative to calamus scriptorius.
shown). In a separate set of experiments, we tested whether the failure of L-glutamate to decrease IGP following atropine was due to a “floor effect.” In two animals, a bolus of SNP (50 μg/kg iv) was given after atropine methyl bromide. Following SNP, there was a robust decrease in IGP (1.6 ± 1.1 mmHg). Thus gastric tone had not been fully inhibited following administration of atropine.

Tests for a NANC Pathway From the Intermediate or Rostral DMV: Methods Used by Other Investigators

To further our investigation, we attempted to repeat the studies of other investigators who demonstrated evidence of a NANC pathway originating from the DMV. In our studies, we included the ipsilateral vagotomy technique to distinguish between effects evoked from the mNTS vs. those evoked from the DMV.

Studies with a low dose of L-glutamate. Raybould and colleagues (36) microinjected 1–10 nl of 0.1 mM to 10 mM L-glutamate into what they collectively called the dorsal vagal complex (NTS plus DMV) of the rat and studied its effects on gastric motility. It was reported that L-glutamate microinjection resulted in only one response: inhibition of gastric motor activity. This response was abolished by bilateral cervical vagotomy. The authors state that “microinjection of GLU only decreased motor activity of the gastric corpus; no site was found at which injection of GLU increased motor activity.”

In four rats, we unilaterally microinjected 30 nl of 0.3 mM L-glutamate (i.e., 10 pmol) into the DMV. This concentration of L-glutamate was in the range of that tested by Raybould and colleague (36). No effect was observed on gastric motility. In the same experiments, 30 nl of 16.7 mM L-glutamate (i.e., 500 pmol) was microinjected into the same site and always increased gastric motility. These increases in motility were subsequently blocked by ipsilateral vagotomy. Microinjection sites for these four experiments were all located in the intermediate DMV (data not shown).

Studies of substance P. In our studies, we microinjected 30 nl of 4.5 mM substance P (i.e., 135 pmol) into the DMV of eight rats. Before proceeding with substance P injections, we always employed our standard protocol of locating the DMV by microinjecting L-glutamate and observing an increase in gastric motility. In the study that we were attempting to confirm (19), the investigators also used L-glutamate to precisely localize the DMV before microinjecting substance P. In two rats, microinjection of substance P produced a slowly developing increase in IGP (0.8 ± 0.4 mmHg; Fig. 9A). Following ipsilateral vagotomy, repeat microinjection of substance P (and L-glutamate) failed to increase IGP (0.0 ± 0.3 mmHg) (Fig. 9A). In a third rat, microinjection of substance P had no effect on gastric motility. In the same animal, a double dose of the peptide (60 nl of a 4.5 mM solution; i.e., 270 pmol) still had no effect. In a fourth rat, we observed a decrease in
gastric tone upon microinjection of substance P into the DMV. This decrease was blocked by ipsilateral vagotomy. In four other DMV experiments, microinjection of substance P produced a decrease in IGP (−1.0 ± 0.3 mmHg, P < 0.05) that was not blocked by ipsilateral vagotomy. However, subsequent contralateral vagotomy abolished the substance P-induced decreases in IGP. In these same experiments, ipsilateral vagotomy countered the increase in IGP evoked by our standard dose of 16.7 mM L-glutamate (+0.2 ± 0.1 mmHg after ipsilateral vagotomy).

Substance P (30 nl of a 4.5 mM solution) was also microinjected into the mNTS of two rats. Again, we used our standard protocol of locating the mNTS by microinjection and observed a decrease in gastric motility before proceeding with substance P microinjections. In both animals, substance P produced a decrease in IGP. Ipsilateral vagotomy did not block the responses to substance P (or to L-glutamate), but subsequent contralateral vagotomy was performed and counteracted the response to substance P.

In summary, histological evidence provided by the microinjection sites (data not shown, but available upon request) indicate that we successfully microinjected substance P into the DMV of eight rats. In only one of the eight rats studied did we observe an inhibition of gastric motility from the DMV that was abolished by ipsilateral vagotomy. This was the only evidence obtained that supports the existence of a NANC pathway originating from the DMV. In the other seven rats, we observed either an excitatory effect on motility that was blocked by ipsilateral vagotomy, an inhibitory effect that required bilateral vagotomy to be blocked (indicative of a mNTS effect), or no effect despite using a double dose of substance P. We also successfully microinjected substance P into the mNTS of two rats. In both rats, substance P inhibited gastric motility and bilateral vagotomy was required to block the response.

Studies of NMDA. Krowicki and colleagues (21) microinjected 20–30 nl of 10–15 mM NMDA into the intermediate and rostral zones of the DMV (0.5–0.9 mm rostral to obex) of the rat and reported increases in IGP and AUC. We began our studies by microinjecting 30 nl of 10 mM NMDA (i.e., 0.3 nmol) into the DMV of three rats. The DMV was first located by microinjecting 16.7 mM L-glutamate and noting the typical increase in IGP with no consistent effect on cardiorespiratory activity. Next, 30 nl of 10 mM NMDA was microinjected at the same site and caused a multitude of effects: decrease in BP, respiratory arrest, and both a decrease (n = 2) and an increase (n = 1) in IGP. [Note: Krowicki et al. (21) artificially respirated their animals and would not have encountered the problem of respiratory arrest.] The effects observed with 10 mM NMDA suggested to us that the concentration was too high and reached neighboring CNS areas to evoke cardiorespiratory effects. We reduced the concentration to 30 nl of a 0.4 mM solution (i.e., 12 pmol) of NMDA, and five experiments were performed. In three experiments, 0.4 mM NMDA microinjected into the DMV increased IGP, and this increase was counteracted by ipsilateral vagotomy. A representative response from one of these experiments is shown in Fig. 9B. In the fourth animal, NMDA produced a biphasic response. Initially, there was a transient decrease in IGP followed by an increase in IGP. Ipsilateral vagotomy blocked the excitatory component of the motility response. L-NAME (10 mg/kg iv) was tested against the NMDA-induced decrease in IGP that remained after ipsilateral vagotomy and was found to have no effect on the response. In the fifth animal, NMDA microinjected into the DMV produced only a decrease in IGP. Subsequent administration of hexamethonium (15 mg/kg iv) completely blocked this response.

In summary, the predominant response produced by 0.4 mM NMDA microinjected into the DMV was an increase in gastric motility that was counteracted by ipsilateral vagotomy. Any observed decrease in gastric motility was either sensitive to blockade by hexamethonium and/or resistant to blockade by L-NAME. Histological verification of the microinjection sites was made for each experiment (data not shown, but available upon request).

Studies of CRF. Lewis and colleagues (23, 24) microinjected 20 nl of a 1.0 μM solution of CRF (i.e., 20 fmol) into what they describe as the dorsal vagal complex (DVC) of the rat. Concomitant with the CRF microinjection was systemic administration of bethanechol (50 μg/kg bolus then continuous intravenous infusion of 20 μg·kg⁻¹·h⁻¹ for 20 min). The bethanechol was to produce “a supramaximal activation of muscarinic cholinergic receptors” and thereby eliminate the possibility of CRF-induced decrease in gastric motility resulting from withdrawal of a cholinergic excitatory DMV pathway to the stomach. Instead, any CRF-induced decrease in gastric motility would be interpreted as activation of an inhibitory NANC pathway from the DMV to the stomach.

We sought evidence for CRF-induced decreases in gastric motility from the DMV by unilaterally microinjecting 30 nl of 0.67 μM CRF into the DMV while monitoring IGP in rats receiving a continuous intravenous infusion of bethanechol. It was not possible to administer CRF bilaterally because it would preclude the use of ipsilateral vagotomy as a criterion for a selective microinjection into the DMV. Even though our total amount of CRF was one-half that used by Lewis et al. (23) (20 fmol bilaterally), the inhibitory response shown in their study appeared robust enough (~83% reduction in gastric motility) for us to detect an effect of CRF.

Four experiments were performed. In three animals, L-glutamate microinjected into the DMV prior to bethanechol increased IGP while repeat microinjection of L-glutamate during bethanechol infusion decreased IGP. The latter decrease in IGP was blocked by ipsilateral vagotomy (Note: these three experiments are described in the RESULTS).

Studies Aimed at Unmarking an NANC Pathway From the Intermediate DMV to the Stomach

In the fourth experiment, 16.7 mM L-glutamate increased IGP before bethanechol but had no effect during bethanechol infusion. In these four animals, microinjection of CRF into the DMV during bethanechol infusion was found to have no significant effect. A tracing of one of these experiments is shown in Fig. 10. In three of the four animals, 0.67 μM CRF was microinjected into the DMV in the absence of bethanechol. Again, no convincing effect on IGP was observed. In one animal, 60 nl of 0.67 μM CRF was tested but still failed to produce any change in gastric motility. Finally, in two of the four rats we also microinjected 30 nl of 0.67 mM CRF (i.e., 20 pmol) unilaterally into the DMV during continuous infusion of bethanechol. At this higher dose,
CRF still did not exhibit an effect on gastric motility. Histological verification of the microinjection sites was made for each experiment (data not shown, but available upon request).

**Studies of oxytocin.** Rogers and Hermann (38) microinjected 2.0 mM oxytocin (2 nl; i.e., 4 pmol) into the DMV of the rat and observed a reduction in gastric motility. In seven animals, we tested three different doses of oxytocin by microinjecting the drug into the DMV. Again, the DMV was first located by microinjecting 16.7 mM L-glutamate. In one animal, 30 nl of 0.067 mM oxytocin (i.e., 2 pmol) was microinjected and did not appear to have a discernible effect on gastric motility. In the other six animals, 30 nl of 0.33 mM oxytocin (i.e., 10 pmol; n = 3) and 30 nl of 0.67 mM oxytocin (i.e., 20 pmol; n = 3) were tested. In five of the six animals, spontaneous contractile activity was present prior to microinjection of oxytocin. In one animal, no contractility was present and oxytocin data could not be obtained. Microinjection of the 0.33 mM solution or the 0.67 mM solution of oxytocin produced inhibition of spontaneous contractions (Fig. 9C). This effect of oxytocin is reflected in the change in the AUC of the motility response. The combined data of these five animals indicated that microinjection of oxytocin decreased AUC by −75.1 ± 25.3 mmHg·s (P < 0.05). This decrease in AUC was repeatable after 30 min. After ipsilateral vagotomy, oxytocin still decreased spontaneously occurring contractility by −129.8 ± 38.2 mmHg·s, P < 0.05 (Fig. 9C). Bilateral cervical vagotomy could not be tested on the oxytocin-induced response because sectioning both vagus nerves abolished all spontaneous motility. The inability of ipsilateral vagotomy to prevent the oxytocin effect suggests that microinjection of oxytocin into the DMV evoked an inhibitory effect by diffusing to the NTS. The microinjection sites for these experiments were all located in the DMV (data not shown, but available on request).

**DISCUSSION**

In our study, we reevaluated the effects that stimulation of the DMV would have on gastric motility in the rat. We explored all areas of this nucleus, seeking sites that activate 1) a cholinergic-cholinergic excitatory pathway, 2) a cholinergic-NANC inhibitory pathway, and 3) a nitrergic-NANC inhibitory pathway. Initially, we microinjected 30 nl of a 16.7 mM solution of L-glutamate into the DMV while monitoring gastric motility. The first area studied was the intermediate DMV. Precise location of the intermediate DMV was found by microinjection of L-glutamate into the stereotaxically designated site and noting an increase in IGP. Our findings indicated that reproducible increases in IGP could be obtained from this region of the DMV. These increases were not associated with any significant changes in BP or heart rate and were completely prevented by performing ipsilateral vagotomy.

Using the L-glutamate-induced increase in IGP as a “locator” of our site made it unlikely that we would find evidence of a cholinergic-NANC inhibitory pathway or a nitrergic-NANC inhibitory pathway in the intermediate DMV. Hence, we tried to unmask such sites by repeating L-glutamate microinjection in animals treated with L-NAME, atropine, or infused with bethanechol. No evidence of inhibitory DMV pathways was obtained in L-NAME-treated (n = 4), atropine-treated (n = 6), or in six of nine bethanechol-infused rats. In three bethanechol experiments, L-glutamate microinjection did produce a decrease in IGP that was prevented by ipsilateral vagotomy. Thus the bulk of our findings obtained from the intermediate DMV indicate that these neurons are the origin of a cholinergic-cholinergic excitatory pathway. However, data obtained from three (out of nine) rats during maximal stimulation of gastric smooth muscle muscarinic receptors indicate the presence of an inhibitory DMV pathway. Presumably, this was a cholinergic-NANC inhibitory DMV pathway because nitrergic-NANC neurons do not appear to be located in the intermediate DMV (20).

In widening our exploration of the DMV, we microinjected L-glutamate into the rostrolateral DMV, an area described as containing a cholinergic-NANC inhibitory pathway (39). This portion of the DMV lies 0.6 to 1.0 mm lateral to the midline and encompasses a caudal-rostral distance from 0.4 to 0.9 mm in front of CS. In these experiments, we relied solely on stereotaxic coordinates to localize the micropipette tip in the designated region of the DMV. That is, L-glutamate was not used as a “locator” for the micropipette tip site. In the 11 experiments performed, only one experiment suggested the presence of a DMV cholinergic-NANC inhibitory pathway in the rostrolateral DMV. In this experiment, L-glutamate microinjection consistently decreased IGP, and this response was not accompanied by any significant change in mean BP or heart rate. More importantly, ipsilateral vagotomy prevented the response. In the other 10 experiments, no evidence for the presence of a cholinergic-NANC inhibitory pathway was found.

Reports indicating the presence of NOS-containing preganglionic neurons located +0.7 to +1.1 mm anterior to CS (19, 21) prompted us to do microinjection studies in the rostral DMV. L-glutamate microinjected into the rostral DMV produced an increase in gastric motility in all of the five animals studied. This increase was prevented by ipsilateral vagotomy.
Last, L-glutamate microinjection studies were performed in the caudal DMV (i.e., DMV caudal to CS). Five successful experiments were conducted. In each case, L-glutamate microinjection produced a decrease in gastric motility, and this decrease was prevented by ipsilateral vagotomy. Pretreatment with L-NAME, a NOS inhibitor, did not counteract the L-glutamate-induced decrease in gastric motility elicited from the caudal DMV. We also observed that the effects of 16.7 mM L-glutamate microinjected into the caudal DMV parallel ongoing studies in the ferret, in that it produces a decrease in fundus contractility. This response is prevented by ipsilateral vagotomy but is unaffected by intravenous L-NAME (M. Niedringhaus, R. Gillis, S. R. T. Evans, P. G. Jackson and N. Sahibzada, unpublished data).

Both the rostral and caudal DMV are areas reported to have NOS-containing preganglionic neurons (19–21, 50). Since L-glutamate microinjected into the rostral portion of the DMV does not produce gastric inhibition, and because microinjection of L-glutamate into the caudal portion of the DMV produces a decrease in gastric motility that is resistant to blockade by L-NAME, we conclude that a functional nitrergic-NANC inhibitory DMV pathway does not exist in the rat.

Our findings obtained from studies of the caudal DMV in the rat confirm our earlier findings obtained in another species, the cat (41). In the earlier study, microinjection of L-glutamate into the caudal DMV produced a statistically significant decrease in stomach pressure (−7.5 ± 2.2 mmHg, n = 4). After ipsilateral vagotomy, L-glutamate microinjection failed to decrease stomach pressure. Studies from two other laboratories using L-glutamate microinjected into the caudal DMV of the rat describe decreases in gastric motility (12, 18), but neither group of investigators tested the effect of ipsilateral vagotomy on the response.

In summary, in our reevaluation of the effects that stimulation of the DMV would have on gastric motility in the rat we found 1) no evidence of a DMV nitrergic-NANC inhibitory pathway, 2) only a few isolated instances in which a cholinergic-NANC inhibitory pathway is present in areas of the DMV rostral to CS (4 out of 43 experiments); and 3) confirmation of our earlier findings of the existence of a cholinergic-NANC inhibitory pathway present in the caudal DMV (41). It is conceivable that our inability to observe a cholinergic-NANC inhibitory pathway in more than 90% of the experiments performed in areas rostral to CS is due to the possibility that the inhibitory pathway is maximally active and could not be further activated by L-glutamate microinjected into the DMV. This possibility will be assessed in future studies. Most importantly, our data obtained with intravenous L-NAME indicate that the DMV cholinergic inhibitory pathway in the DMV caudal to CS does not utilize NO as a neurotransmitter.

The present data, combined with our earlier data (5, 6, 42) and the findings of other investigators (13, 16) indicate, at best, a minor role for the caudal DMV inhibitory pathway in the brain control of gastric motility. Evidence for this is that methods used to inhibit gastric motility in rat (e.g., esophageal distention, intravenous glucose, CCK, and nicotine infusion) have demonstrated a lack of c-Fos activation in the caudal DMV of the rat (6, 7, 42). Furthermore, studies of a vago-vagal reflex involving the superior laryngeal nerve showed that activation of water-responsive afferents led to inhibition of gastric motility, but not inhibition of gastric motility because neurons of the mNTS project to both the ipsilateral and contralateral DMV. This has been reported in studies using pseudorabies virus (PRV) injected into the ventral
stomach of the rat (37). After postinjection survival times of 60–64 h (stage 3 infection), PRV immunolabeling was observed bilaterally in the medial subnuclei of the NTS.

The implication of our findings is that a prerequisite for knowing whether microinjections of a drug have been made specifically in the DMV is to perform ipsilateral vagotomy. Blockade of a response by bilateral vagotomy does not distinguish between action of the drug at the DMV or the NTS. This emphasis on the importance of ipsilateral vagotomy draws attention to the fact that the published data concluding the presence of a DMV cholinergic and/or nitrergic NANC gastric inhibitory pathway are based on results obtained after bilateral vagotomy (19, 21, 23, 29, 38).

Since we had delineated a functional method by which to characterize DMV responses and differentiate them from NTS responses, we set out to repeat many of the published studies that based their evidence of a DMV gastric inhibitory pathway on bilateral vagotomy. In the studies of Krowicki and colleagues (19, 21), it was reported that the excitatory agents substance P and NMDA microinjected into the DMV produced a decrease in gastric contractility. This decrease was blocked by bilateral vagotomy. When we performed similar studies, we found that ipsilateral vagotomy never blocked inhibitory responses produced by microinjecting NMDA into the DMV. In the case of substance P, we observed blockade of inhibition by ipsilateral vagotomy in only one of eight rats. Instead, in all of the NMDA studies and in seven of eight rats of the substance P studies, bilateral vagotomy was required to abolish inhibitory responses, suggesting that these substances reached the NTS by diffusion from sufficiently high concentrations. In some cases, microinjection of substance P or NMDA actually increased gastric motility. This response was blocked by bilateral vagotomy, suggesting a DMV effect.

Regarding oxytocin, Rogers and Hermann (38) reported that microinjection of this neuropeptide into the DMV produces a reduction in gastric motility and excitations from the NTS of the rat, the threshold dose of L-glutamate has been reported to be 25 nl of a 3 mM solution (44). On the basis of our findings with 0.3 mM L-glutamate, the findings of Ferreira (4) and the findings of Spencer and Talman (44), we conclude that the concentration of L-glutamate in 30 nl of a 0.3 mM solution (i.e., 10 pmol) is too low to produce a change in gastric motility from the DMV.

Our microinjection studies with CRF are also suggestive of a drug dose that is too low. CRF (30 nl of a 0.67 μM solution) was unilaterally microinjected into the DMV during continuous bethanechol infusion. In one animal, we tried 60 nl of 0.67 μM CRF and studied its effects on spontaneous gastric motility. Neither dose of CRF produced an effect on gastric motility. This contrasts with the findings of Lewis et al. (23), who reported robust inhibitory effects from bilateral microinjection of 20 nl of 1.0 μM CRF (i.e., 20 fmol) during bethanechol infusion. In two animals, we unilaterally microinjected 30 nl of 0.67 mM CRF during bethanechol infusion and observed no distinct inhibitory effect of this dose. It should be mentioned that we used a low-compliance balloon recording technique to monitor gastric motility, whereas Lewis and colleagues used a strain gauge technique. In an earlier study, we found that the balloon recording method is more sensitive for detecting global changes in gastric motility (6). Hence, if CRF in the femtomole dose range were to exert an effect, we should have been able to observe it in the IGP trace. The lack of an effect in our study compelled us to question the rationale for using such a small dose of drug. Lewis et al. (23) report dose-response data for an excitatory effect of CRF on DMV neurons in the rat brain slice preparation. The data indicate that 30–100 nM are effective in exciting these neurons. In another recent in vitro dose-response study of substance P on rat DMV neurons, the effective concentration of this agent was 1 μM (24). Thus CRF is ~10 times more potent than substance P in exciting DMV neurons. Dose-response data for substance P on gastric motility changes (obtained via microinjection into the dorsomedial medulla of the rat) indicate that a dose in the range of 135–405 pmol is an effective excitant (19). Thus we found it more suitable to try one-tenth this dose of CRF (i.e., 30 nl of a 0.67 mM solution; 20 pmol total) for microinjection studies. Indeed, others who have reported DVC effects of CRF have used a dose range of 63–210 pmol (i.e., 75 nl of a 0.84–2.8 mM solution) (14).

Additional evidence that CRF does not excite DMV neurons can be found in two other published reports. In one report using c-Fos as a marker of neuronal activation, CRF administration
increased c-Fos expression in the NTS and AP of rats but did not increase c-Fos expression in the DMV (27). In the other report, intracisternal injection of CRF in rats suppressed multiunit efferent activity recorded from gastric vagal fibers (48).

In reassessing the effect of 30 nl of 0.67 μM CRF (i.e., 20 fmol) in the DMV of rats, in studying the functionally effective doses of CRF, and in assessing the literature for evidence of excitatory effects of CRF on DMV neurons, we are compelled to conclude that CRF (at this dose) does not activate a NANC pathway originating from the DMV. Our inability to confirm the findings of Lewis and colleagues (23) forces us to further conclude that a careful dose-response study of this neuropeptide needs to be carried out at the DMV.

Regarding the issue of excessively high doses of microinjected drug as a confounder for data interpretation, we refer to the NMDA study of Krowicki et al. (21), in which the dose of NMDA in that study was so high (20–30 nl of a 10–15 mM solution of NMDA) that conclusions drawn may be inappropriate. When we microinjected 30 nl of 10 mM NMDA into the DMV, we observed widespread hindbrain effects. Most noticeably, the animals experienced respiratory arrest and required artificial respiration. To avoid these widespread effects, we reduced the dose to 0.4 mM NMDA. A review of the literature indicated that NMDA is ~30 times more potent than L-glutamate (22), and 50 nl of a 0.14 mM solution of NMDA is sufficient to evoke robust cardiovascular responses when microinjected into the dorsomedial hypothalamus of the rat (43).

As a result of our reevaluation of the effects that stimulation of the DMV has on gastric motility, we can draw the following conclusions: 1) specific regions of the DMV are described to have a significant number of preganglionic vagal neurons that comprise an inhibitory pathway. Studies of L-glutamate microinjected into these three regions of the DMV (intermediate, rostral, and rostrolateral DMV) indicated primarily the presence of a cholinergic-cholinergic excitatory pathway in the 43 experiments performed; 2) studies of a fourth area of the DMV (the caudal DMV) confirmed earlier findings obtained in the cat (41) regarding the existence of an inhibitory DMV pathway to gastric smooth muscle. The inhibitory pathway does not appear to use nitric oxide as a neurotransmitter; 3) no evidence was obtained for the existence of a DMV nitricergic-NANC inhibitory pathway; 4) comparison of gastric motility responses obtained from the DMV and the mNTS and comparison of the effect of vagotomy on these responses indicated that a test of ipsilateral vagotomy is required to distinguish between effects originating from the DMV and effects originating from the mNTS; and 5) attempts to repeat previous microinjection studies that have provided evidence of a DMV-NANC inhibitory pathway indicate that the findings of other investigators are open to question. This is because diffusion of injectate from DMV to the NTS probably occurred and because doses of the drugs were either too low or too high for studying effects specifically at the DMV.

GRANTS

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