Dorsal motor nucleus of the vagus: a site for evoking simultaneous changes in crural diaphragm activity, lower esophageal sphincter pressure, and fundus tone

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Niedringhaus M, Jackson PG, Evans SR, Verbalis JG, Gillis RA, Sahibzada N. Dorsal motor nucleus of the vagus: a site for evoking simultaneous changes in crural diaphragm activity, lower esophageal sphincter pressure, and fundus tone. Am J Physiol Regul Integr Comp Physiol 294: R121–R131, 2008. First published October 31, 2007; doi:10.1152/ajpregu.00391.2007.—The sphincter mechanism at the esophagogastric junction is comprised of both smooth muscle of the lower esophagus and the skeletal muscle of the crural diaphragm (CD) (1, 7, 25, 27, 28). Together, they contribute to the competency of the lower esophageal high-pressure zone (26), which serves as an antireflux barrier. In reflux disease, the competency of this barrier is compromised by increased occurrence of simultaneous relaxation of the smooth muscle of the lower esophagus and inhibition of the CD (26). To understand this phenomenon, it is necessary to elucidate how these two muscles are controlled. In this regard, it is known that a major source of innervation of the smooth muscle comprising the lower esophagus is the dorsal motor nucleus of the vagus (DMV) (1, 4, 29, 35, 43). However, to our knowledge, no published data are available on the location of a brain site or sites that exert control over the other component of sphincter function, the CD.

In considering the likely site (or sites) in the hindbrain that provides control over the CD, we focused our attention on the DMV. This hindbrain nucleus is a key component of vagovagal reflexes (42), and activation of a vagovagal reflex has been shown to inhibit the CD (13). In support of this approach to finding the brain site or sites for CD control, Yates’s et al. (46) reported that injections of the retrograde transneuronal tracer, pseudorabies virus (PRV) into the diaphragm of the ferret-labeled neurons in the DMV, including both the CD and the costal diaphragm. They interpreted their findings as due to leakage of PRV from the injection site into the peritoneal cavity; however, they did not rule out the possibility that DMV neurons were labeled because of transneuronal retrograde transport from the CD.

Recently, we reported preliminary data that PRV injected into the CD of the ferret retrogradely labels neurons in the DMV (16). Control injections of PRV into the abdominal space around the CD failed to label cells in the DMV (20), suggesting that the projections to the CD are not due to leakage of tracer into the peritoneal cavity. Subsequent experiments with the retrograde monosynaptic tracer cholera toxin B have since confirmed the direct nature of this projection from the DMV to the CD (M. Niedringhaus, P. G. Jackson, R. Pearson, M. Shi, K. Dretchen, R. A. Gillis, and N. Sahibzada, unpublished data). Young et al. (47, 48) have also presented preliminary data showing that the vagus nerve directly innervates the CD in the ferret. Following injections of cholera toxin B into the CD, these authors reported retrogradely labeled neurons in the DMV that were immunopositive for choline acetyltransferase.

Based on the above observations, the first aim of our study was to test the hypothesis that excitation of neurons in the DMV would result in changes in CD activity (i.e., bursts/min) were prevented by ipsilateral vagotomy. Our experiments were performed on anesthetized male ferrets whose LES pressure, CD activity, and fundus tone were monitored. To activate DMV neurons, L-glutamate was microinjected unilaterally into the DMV at three sites: intermediate, rostral, and caudal. Stimulation of the intermediate DMV decreased CD activity (−4.8 ± 0.1 bursts/min and −0.3 ± 0.01 mV) and LES pressure (−13.2 ± 2.0 mmHg; n = 9). Stimulation of this brain site also produced an increase in fundus tone. Stimulation of the rostral DMV elicited increases in the activity of all three target organs (n = 5). Stimulation of the caudal DMV had no effect on the CD but did decrease both LES pressure and fundus tone (n = 5). All changes in LES pressure, fundus tone, and some DMV-induced changes in CD activity (i.e., bursts/min) were prevented by ipsilateral vagotomy. Our data indicate that simultaneous changes in activity of esophagogastric sphincters and fundus tone occur from rostral and intermediate areas of the DMV and that these changes are largely mediated by efferent vagus nerves.

acetylcholine; nitric oxide; vagus; vasoactive intestinal polypeptide

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Based on the above observations, the first aim of our study was to test the hypothesis that excitation of neurons in the DMV would result in changes in CD activity (i.e., bursts/min) were prevented by ipsilateral vagotomy. Our experiments were performed on anesthetized male ferrets whose LES pressure, CD activity, and fundus tone were monitored. To activate DMV neurons, L-glutamate was microinjected unilaterally into the DMV at three sites: intermediate, rostral, and caudal. Stimulation of the intermediate DMV decreased CD activity (−4.8 ± 0.1 bursts/min and −0.3 ± 0.01 mV) and LES pressure (−13.2 ± 2.0 mmHg; n = 9). Stimulation of this brain site also produced an increase in fundus tone. Stimulation of the rostral DMV elicited increases in the activity of all three target organs (n = 5). Stimulation of the caudal DMV had no effect on the CD but did decrease both LES pressure and fundus tone (n = 5). All changes in LES pressure, fundus tone, and some DMV-induced changes in CD activity (i.e., bursts/min) were prevented by ipsilateral vagotomy. Our data indicate that simultaneous changes in activity of esophagogastric sphincters and fundus tone occur from rostral and intermediate areas of the DMV and that these changes are largely mediated by efferent vagus nerves.

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been suggested to be mediated in the rat by changes in vagus efferent activity to the stomach consisting of parallel excitatory and inhibitory nonadrenergic, noncholinergic (NANC) pathways (14, 23, 33, 34). This NANC pathway is described as comprised of preganglionic DMV neurons synapsing onto nitric oxide (NO)-releasing enteric neurons (14, 23, 40). We and another group of investigators (8, 20) have not been able to obtain evidence of functional parallel excitatory and inhibitory DMV vagal pathways to the smooth muscle of the stomach in the rat. Thus, an aim of our study was to test the hypothesis that DMV stimulation in the ferret at a site that evokes changes in LES pressure would simultaneously affect fundus tone in at least 50% of the studies and that the fundus tone effect would not involve nitricergic transmission as suggested by others in the rat (14, 23).

**METHODS**

All experimental procedures conformed to the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and were approved by the Georgetown University Institutional Animal Care and Use Committee.

**Animals and surgical preparation.** Adult male ferrets (*Mustela putorius furo*) weighing 600–700 g (Marshall Farms, NY) were housed in controlled conditions at room temperature (22°C) and light (12:12-h light-dark cycle) with free access to food and water. Before each experiment, food was withheld overnight, whereas water was provided ad libitum. Each animal was initially sedated with an intramuscular injection of ketamine (25 mg/kg). Each animal was then exposed to isoflurane via a nose cone (4% induction; 1.5% maintenance) to obtain a lack of response to a toe pinch and a lack of corneal reflex. After induction of anesthesia, the nose cone was then switched with an intubation tube subsequent to a tracheotomy.

After induction of anesthesia, the carotid artery and the jugular vein were cannulated for monitoring blood pressure and for systematically administering drugs, respectively. The cervical vagus nerve trunk on both sides of the neck were carefully isolated and encircled with a saline-soaked suture. Blood pressure was monitored by a pressure transducer that was connected via a bridge amplifier to a data acquisition system (PowerLab; ADI Instruments, Colorado Springs, CO). Body temperature was monitored by a rectal thermometer and main-
so that the elicited responses were entirely from the DMV. All successful L-glutamate microinjections were repeated at least once after a 10-min interval to demonstrate the reproducibility of the response at a time of full recovery. The dose of 500 pmol/30 nl was taken from the dose-response data of Ferreira (9), who reports its location toward the top of the curve but not lying in the plateau of the response. In addition, this dose is lower than the dose range (>5 nmol) shown to exert a depolarization-induced blockade of neurons in the immediate vicinity of the pipette tip (24). Furthermore, this dose is in the same range as two other studies that have examined the effects of L-glutamate-induced excitation in the CNS on gastric motility (9, 38). Finally, it is severalfold less than the dose used by Abrahams, et al. (1) in the ferret.

After establishing the effects of L-glutamate microinjection into the DMV on gastrointestinal (GI) responses, the effects of other experimental manipulations were studied on these responses.

**Experimental protocols.** For studying the effect of ipsilateral vagotomy on the L-glutamate response, at least two responses to microinjection of L-glutamate (500 pmol/30 nl) into the DMV were obtained. Next, the ipsilateral cervical vagus was severed. A stabilization period of 10 to 15 min ensued before L-glutamate microinjection was repeated into the DMV. The effects of ipsilateral vagotomy on DMV- and NTS-induced responses of the GI tract have been extensively studied by us (11). Ipsilateral vagotomy was performed at the end of each experiment in all protocols to ensure the location of the pipette and to demonstrate the vagal nature of our elicited responses.

For studying the effect of N\(^5\) nitro-L-arginine methyl ester (L-NAME) on the L-glutamate response, two reproducible responses to L-glutamate were first obtained. Next, L-NAME (10 mg/kg iv), an NO synthase (NOS) inhibitor, was administered to assess the contribution of NO on the target tissues vis-a-vis the NANC vagal pathway. The dose used is one that has been used by others (14, 23, 40, 41) and us (8) to block vagal-induced relaxatory responses from the GI tract. After an interval of 5 to 10 min following L-NAME administration, the L-glutamate microinjection was repeated. Similar to the L-NAME protocol, the vasoactive intestinal polypeptide (VIP) receptor antagonist (\(\alpha\)-P-CI-Phe\(^6\),Leu\(^{17}\))-VIP (VIP\(\alpha\)) was administered (0.35 mg/kg iv) 5 to 10 min prior to L-glutamate microinjection into the DMV. The dose used of VIP\(\alpha\) is comparable with that shown to be effective for blocking VIP-related GI effects (6).

For studying the effects of both L-NAME and VIP\(\alpha\) on the L-glutamate response, two reproducible responses to L-glutamate were first obtained. Next, each animal received a sequential intravenous administration of either L-NAME, then VIP\(\alpha\) or VIP\(\beta\), and then L-NAME. Five to 10 min later, L-glutamate microinjection was made into the DMV and from thereon at intervals of 10 min until signs of recovery of the responses were observed (typically within 30–40 min).

Finally, for studying the effects of atropine methyl bromide on the L-glutamate response, the drug was administered intravenously in a dose of 0.1 mg/kg. The dose of atropine was chosen based on its ability to fully block muscarinic receptors in the periphery (12). Atropine methyl bromide was selected because it is a permanently charged molecule and does not cross the blood-brain barrier.

**Data analysis.** Data were analyzed using Chart software (AD Instruments). Values for each experimental endpoint were divided into two values, baseline and during L-glutamate. Baseline values for the LES pressure were defined to be the average barrier pressure of the LES over a 2-min recording period prior to microinjection of L-glutamate. The during L-glut pressure was defined to be the largest magnitude of change in the barrier LES pressure within the time course of action of L-glutamate. The time course of the response was divided into two values: the time to peak and the duration. The time to peak was defined as the time (in seconds) between the initiation of the L-glutamate-induced response (typically within the time during which L-glutamate was being administered) and the point where during L-glutamate value was recorded. The duration was defined as the time (in seconds) between the initiation of the L-glutamate response and the point where the LES pressure returned to equivalent baseline value. Since in all animals, L-glutamate was administered...
Table 1. Effects of L-GLU microinjected into 3 areas of the DMV on CD activity, LES pressure, and fundus tone before and after IpsiVx

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Baseline</th>
<th>During L-GLU</th>
<th>Baseline</th>
<th>During L-GLU</th>
<th>Baseline</th>
<th>During L-GLU</th>
<th>Baseline</th>
<th>During L-GLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.8 ± 1.4</td>
<td>−4.8 ± 0.1 *</td>
<td>0.4 ± 0.1</td>
<td>−0.3 ± 0.01 *</td>
<td>33.4 ± 3.1</td>
<td>−13.2 ± 2.0 *</td>
<td>5.2 ± 0.3</td>
<td>+0.7 ± 0.10 *</td>
</tr>
<tr>
<td>After IpsiVx</td>
<td>15.4 ± 1.1</td>
<td>−0.7 ± 0.9 †</td>
<td>0.5 ± 0.04</td>
<td>−0.2 ± 0.03 *</td>
<td>32.5 ± 3.0</td>
<td>+0.3 ± 0.36 †</td>
<td>5.1 ± 0.3</td>
<td>+0.03 ± 0.02 †</td>
</tr>
</tbody>
</table>

**Intermediate DMV**

| Control                | 21.5 ± 1.9 | +6.5 ± 2.3 * | 0.6 ± 0.16 | +0.05 ± 0.04 | 31.0 ± 2.4 | +15.9 ± 3.4 * | 5.0 ± 0.5 | +1.0 ± 0.20 * |
| After IpsiVx           | 16.6 ± 2.3 | +0.34 ± 0.23 † | 0.7 ± 0.16 | +0.2 ± 0.06 | 26.9 ± 3.5 | +0.3 ± 0.38 † | 4.4 ± 0.5 | −0.02 ± 0.06 † |

**Rostral DMV**

| Control                | 15.7 ± 0.4 | −0.2 ± 0.5 | 0.3 ± 0.04 | −0.00 ± 0.01 | 26.0 ± 2.8 | −7.9 ± 1.1 * | 5.8 ± 0.8 | −0.9 ± 0.3 * |
| After IpsiVx           | 11.5 ± 0.9 | −0.4 ± 0.2 | 0.4 ± 0.04 | −0.01 ± 0.01 | 26.0 ± 3.3 | −0.2 ± 0.2 † | 4.7 ± 0.7 | −0.10 ± 0.10 † |

**Caudal DMV**

Data expressed as means ± SE; n = 9 ferrets in each group receiving intermediate DMV and 5 ferrets in each group receiving rostral or caudal DMV. DMV, dorsal motor nucleus of the vagus; CD, crural diaphragm; LES, lower esophageal sphincter; IpsiVx, ipsilateral vagotomy; L-GLU, L-glutamate. *One-sample t-test and †paired t-test comparing magnitude of change before IpsiVx with change after IpsiVx (significant at P < 0.05).
Table 2. Effects of L-NAME (10 mg/kg iv) and L-NAME + VIPa (0.35 mg/kg iv) on the time course of the decrease in LES pressure produced by L-GLU microinjected unilaterally into the intermediate part of the DMV

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Time-to-Peak Decrease in LES Pressure</th>
<th>Duration of Decrease in LES Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.4±1.9</td>
<td>127±8</td>
</tr>
<tr>
<td>After L-NAME</td>
<td>37.8±6.8*</td>
<td>221±34*</td>
</tr>
<tr>
<td>Control</td>
<td>14.0±1.9</td>
<td>126±13</td>
</tr>
<tr>
<td>After VIPa</td>
<td>4.0±0.6*</td>
<td>42±13*</td>
</tr>
</tbody>
</table>

Data are expressed in seconds as means ± SE; n = 4 ferrets per group. L-NAME, N\(^\text{-}\)nitro-L-arginine methyl ester; VIPa, vasoactive intestinal polypeptide (VIP) receptor antagonist, (o-P-Chl-Phe\(^\text{b}\),Leu\(^{17}\))-VIP. *Paired t-test (significant at P < 0.05).

Regent Laboratories (Shirley, NY); L-glutamate, L-NAME and atropine methyl bromide were obtained from Sigma-Aldrich (St. Louis, MO); and (o-P-Chl-Phe\(^{b}\),Leu\(^{17}\))-VIP was purchased from Tocris (Ellisville, MO).

RESULTS

Effects produced by microinjection of L-glutamate into the intermediate area of the DMV. L-glutamate (500 pmol/30 nl) was microinjected unilaterally into the intermediate area of the DMV of nine ferrets. End points measured were CD EMG (amplitude and frequency), intraluminal pressure of the lower esophageal sphincter (LES), fundus tone, mean arterial blood pressure, and heart rate. L-glutamate produced a decrease in EMG activity of the CD (both amplitude and frequency decreased), as well as a decrease in LES pressure. In addition, L-glutamate microinjection produced an increase in the tone of the fundus. These results appear in Table 1. L-Glutamate microinjection did not affect mean blood pressure or heart rate (data not shown).

An example of the effects of L-glutamate microinjection appears in Fig. 1. The location of the microinjection site for this experiment is shown (Fig. 1A) as well as microinjection sites for all nine ferrets studied (Fig. 1C). There are decreases in the EMG activity and LES pressure, and an increase in the tone of the fundus.

The time to peak of all of these changes ranged from ~15 s (CD) to 40 s (LES), and these changes lasted about 30 s for the CD and 4 min for the LES and fundus.

In the nine animals reported in Table 1, the effects of ipsilateral vagotomy on all the end points measured were evaluated. The data indicate that ipsilateral vagotomy prevented unilateral microinjection of L-glutamate from decreasing EMG frequency of the CD and LES pressure. Ipsilateral vagotomy also prevented unilateral microinjection of L-glutamate from increasing the tone of the fundus. On the other hand, ipsilateral vagotomy did not prevent unilateral microinjection of L-glutamate from decreasing EMG amplitude of the CD. The experimental traces in Fig. 1C, right illustrate the effects of ipsilateral vagotomy.

In addition to the nine experiments described above, we performed two types of control experiments. In four animals, we purposely microinjected L-glutamate unilaterally into medullary areas just outside the DMV. In an additional three animals, we microinjected saline vehicle (pH 7.2–7.4; equivalent to the pH of the L-glutamate solution) unilaterally into the DMV. In both control groups, no significant effects were observed on EMG activity of the CD, LES pressure, and on fundus tone following microinjection. The microinjection sites for the control experiments where we purposely microinjected L-glutamate just outside the DMV are shown in Fig. 1B (C). As can be noted, two of the sites outside the DMV were in the nucleus tractus solitarius (NTS), whereas the other two were in the hypoglossal nucleus. We have previously reported that NTS stimulation effects the esophageal sphincters (37); however, to do so, the micropipette needs to be in the medial NTS. Our present control injections into the NTS were in the dorsomedial part of this nucleus.

The peripheral neurotransmitter(s) responsible for relaxation of the LES sphincter was sought by determining whether an inhibitor of NOS, L-NAME (10 mg/kg iv), would alter the response. The magnitude of the decrease in LES pressure evoked by L-glutamate microinjection was unchanged by L-

Table 3. Effects of L-GLU microinjected into 3 areas of the DMV on CD activity, LES pressure, and fundus force before and after atropine methyl bromide

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Burst/min</th>
<th>Amplitude, mV</th>
<th>LES Pressure, mmHg</th>
<th>Fundus Force, g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>During L-GLU</td>
<td>Baseline</td>
<td>During L-GLU</td>
</tr>
<tr>
<td>Intermediate DMV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.9±1.4</td>
<td>−6.4±2.3*</td>
<td>0.5±0.02</td>
<td>−0.3±0.12*</td>
</tr>
<tr>
<td>After atropine methyl bromide</td>
<td>17.9±1.1</td>
<td>−7.4±3.0*</td>
<td>0.5±0.06</td>
<td>−0.1±0.14</td>
</tr>
<tr>
<td>Rostral DMV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>21.5±3.2</td>
<td>+2.6±0.2*</td>
<td>0.4±0.03</td>
<td>+0.06±0.04</td>
</tr>
<tr>
<td>After atropine methyl bromide</td>
<td>21.8±2.7</td>
<td>+3.0±1.4*</td>
<td>0.4±0.04</td>
<td>+0.08±0.04</td>
</tr>
<tr>
<td>Caudal DMV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.8±2.3</td>
<td>+0.2±0.2</td>
<td>0.3±0.03</td>
<td>+0.01±0.01</td>
</tr>
<tr>
<td>After atropine methyl bromide</td>
<td>16.7±2.8</td>
<td>−0.1±0.2</td>
<td>0.4±0.03</td>
<td>+0.03±0.01</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 3 ferrets in each group receiving intermediate and rostral DMV and 2 ferrets in each group receiving caudal DMV. *One-sample t-test and †paired t-test comparing magnitude of change before atropine methyl bromide with magnitude of change after atropine methyl bromide (significant at P < 0.05).
NAME pretreatment (Fig. 2A). L-NAME pretreatment did change the time course of the response. Both the time to reach the nadir of the response and the duration of the response were altered (Table 2). Prior to L-NAME, the time to reach the nadir was 16.4 ± 1.9 s. After L-NAME, the time to reach nadir was significantly increased (Table 2). L-NAME pretreatment had no effect on either the magnitude of changes in EMG activity of the CD (Fig. 2A), increase in tone of the fundus (data not shown), or their time courses (data not shown). L-NAME pretreatment per se produced an initial increase in the baseline values for LES pressure (+4.3 ± 0.9 mmHg; \( P < 0.05 \)); this increase was observed 2 min after L-NAME administration but was not maintained.

Due to the lack of effect of L-NAME on the magnitude of the decrease in LES pressure, we next determined the effect of VIPa on this response. Pretreatment with VIPa (0.35 mg/kg iv) did not reduce the magnitude of the LES relaxation in response to L-glutamate microinjection into the DMV (Fig. 2B) but did significantly shorten the time it took the response to reach nadir (Table 2). After VIPa administration, the duration of the response was significantly reduced (Table 2). VIPa pretreatment had no effect on either the magnitude of changes in EMG activity of the CD (data not shown), or their time courses (data not shown). Finally, VIPa pretreatment per se produced no significant changes in the baseline values for LES pressure (Fig. 2B).

Since neither L-NAME nor VIPa reduced the magnitude of the decrease in LES pressure evoked by microinjection of L-glutamate, we next tested the two drugs in combination on the response. These data are tabulated in Fig. 2, A and B, and indicate that pretreatment with the combination of L-NAME and VIPa prevents the decrease in LES pressure evoked by L-glutamate microinjection into the intermediate area of the DMV.

Similar to pretreatments with L-NAME and VIPa alone, the combination had no effect on either the magnitude of changes in EMG activity of the CD, LES pressure, and fundus tone.
in EMG activity of the CD or tone of the fundus (data not shown) that were evoked by L-glutamate microinjection or their time courses (data not shown). Additionally, the combination produced no significant changes in baseline values for LES pressure (Figs. 2, A and B).

We also evaluated the effects of atropine methyl bromide on L-glutamate-induced excitation of the intermediate DMV (Table 3). Atropine methyl bromide blocked the increase in fundus tone, but not the decreases in LES pressure and EMG activity (i.e., decrease in frequency of bursts) of the CD. However, atropine methyl bromide did appear to prevent the L-glutamate-induced decrease in the amplitude of the CD. Atropine methyl bromide per se produced an initial decrease in the baseline values for LES pressure (−3.5 ± 0.9 mmHg; P < 0.05); this decrease was noted 2 min after atropine methyl bromide was administered but was not maintained.

Effects produced by microinjection of L-glutamate into the rostral area of the DMV. L-glutamate was microinjected into the rostral area of the DMV of five ferrets and produced an increase in frequency of the EMG activity of the CD, LES pressure, and tone of the fundus (Table 1). L-glutamate microinjections did not affect mean blood pressure or heart rate (data not shown).

An example of the effects of L-glutamate microinjection appears in Fig. 3C. There was an increase in the EMG burst frequency of the CD. There was also an increase in the LES pressure and in the fundus tone. The time to peak for the sphincter changes was ~10 to 25 s and lasted an additional 30 to 60 s. The time to peak effect for the fundus was about 20 s and the response duration was ~1 min. The site of microinjection of L-glutamate for this particular experiment appears in Fig. 3A. Microinjection sites for all experiments are depicted in Fig. 3B. In each case, the micropipette tip was located in the rostral area of the DMV. The filled squares represent sites where L-glutamate was administered, and results similar to Fig. 3C were obtained.

The effects of ipsilateral vagotomy on the L-glutamate-induced changes in the experimental end points were also studied. L-glutamate microinjection following ipsilateral vagotomy was unable to produce any effect on the EMG frequency of the CD, on the LES pressure, or on the fundus tone (Table 1; Fig. 3C, right). Control experiments were also performed. Neither saline vehicle microinjected into the DMV (n = 3) nor L-glutamate microinjected outside the DMV (n = 4) had any significant effect. Microinjection sites for these experiments are depicted in Fig. 3B, □.

We also evaluated the effects of atropine methyl bromide on L-glutamate-induced excitation of the rostral DMV (Table 3). Atropine methyl bromide blocked the effects of L-glutamate on both the LES pressure and the fundus tone. However, the increase in the EMG frequency of the CD induced by L-glutamate microinjection was not significantly affected. Atropine methyl bromide per se produced an initial decrease in the baseline values for LES pressure (−3.6 ± 0.2 mmHg; P < 0.05). This decrease was noted ~2 min after the drug was administered but was not maintained.

Effects produced by microinjection of L-glutamate into the caudal area of the DMV. L-glutamate was microinjected into the caudal area of the DMV of five ferrets and produced a decrease in LES pressure and fundus tone. There was no significant effect on the EMG activity of the CD. These results appear in Table 1. In addition, L-glutamate microinjection did not affect mean blood pressure or heart rate (data not shown).

An example of the effects of L-glutamate microinjection appears in Fig. 4B. Following L-glutamate microinjection into the caudal DMV, there were significant decreases in the LES pressure and fundus tone. The time to peak effect of the LES decrease was ~10 s and about 90 s for the fundus; the decrease lasted about 1.5 min for the LES and 2.5 min for the fundus. In all five animals, the effects of ipsilateral vagotomy on the L-glutamate-induced changes were also studied. As can be seen in Fig. 4B (and Table 1), L-glutamate microinjection following
ipsilateral vagotomy was unable to produce any effect on LES pressure or on fundus tone. The location of the microinjection sites for all five animals are shown as Fig. 4A, ■. In two animals, we microinjected L-glutamate into medullary areas just outside the DMV (Fig. 4A, □). No significant effects were found on LES pressure, EMG activity of the CD, and fundus tone.

The peripheral neurotransmitter(s) responsible for relaxation of the LES and fundus was sought by determining whether L-NAME (10 mg/kg iv) would alter the responses. L-glutamate-induced changes in LES pressure and fundus tone were not significantly ($P > 0.05$) altered (Fig. 5A). L-NAME per se produced a significant increase in LES pressure (+2.9 ± 0.4 mmHg; $P < 0.05$), but the increase was not maintained.

We next determined the effect of the VIPa on the DMV-evoked decrease in LES pressure (and decrease in fundus tone) in three ferrets pretreated with L-NAME. Treatment with VIPa (0.35 mg/kg) following L-NAME administration did not alter the effect of L-glutamate microinjection on either LES pressure or fundus tone (Fig. 5B).

The effects of intravenously administered atropine methyl bromide were also evaluated on L-glutamate-induced decreases in LES pressure and fundus tone evoked from the caudal DMV. Atropine methyl bromide treatment ($n = 2$) had no effect on the L-glutamate-induced decrease in LES pressure (Table 3). Its effect on L-glutamate-induced decrease in fundus tone was difficult to interpret. The decrease in fundus tone appeared to be attenuated, but this might have been due to the change in the baseline fundus tone. Fundus tone appeared to be decreased after atropine methyl bromide in these animals, and this lower baseline may have affected the magnitude of the L-glutamate-induced decrease in this tone (Table 3). Finally, atropine methyl bromide per se appeared to decrease the baseline LES pressure ($-3.4 \pm 0.9$ mmHg) but was not statistically significant, which was most likely due to the small number of animals studied ($n = 2$).

Our lack of success in counteracting L-glutamate-induced decrease in LES pressure and fundus tone with L-NAME, L-NAME + VIPa, and atropine methyl bromide led us to test whether combinations of L-NAME + atropine methyl bromide ($n = 3$) and L-NAME + VIPa + atropine methyl bromide ($n = 2$) would antagonize these effects. None of the drug combinations used as pretreatments altered these effects elicited from the caudal DMV.

**DISCUSSION**

In this study, we showed that excitation of rostral and intermediate, but not caudal, DMV produces significant changes in the activity of the CD. These changes were accompanied with alterations in the LES tone. A schematic depicting the functional neurocircuitry associated with these changes is shown in Fig. 6. Focusing on the intermediate DMV, L-glutamate-induced stimulation produced simultaneous decreases in CD activity and in LES pressure that was vagally mediated. Furthermore, our data indicate that after L-NAME treatment, the early drop in LES pressure seen following intermediate DMV stimulation is absent; instead, it now takes longer for LES pressure to reach its nadir. These data point to NO as primarily mediating the decrease in LES pressure that occurs over the first 10–30 s of the response. The later decrease in LES pressure appears to be mediated mainly by VIP. Neither L-NAME or VIPa treatment had any effect on DMV-induced changes in CD activity.

The novel effect of the vagus on CD burst frequency, which was abolished by ipsilateral vagotomy, was not mediated via muscarinic receptors since intravenous atropine methyl bromide pretreatment did not modify the response. Hence, by exclusion, we suggest that the response was mediated by nicotinic receptors located on the CD muscle. The ineffectiveness of atropine in modifying the DMV-induced change in the burst frequency of the CD also suggests that these medullary vagal motoneurons directly innervate the CD. Precedence for this exists for the ciliary muscle of the eye. The ciliary muscle receives a significant part of the parasympathetic innervation directly from midbrain neurons that bypass the ciliary ganglion (17, 45).
Using two recombinants of PRV (PRV-152 and PRV-BaBlu) that were injected into the LES and the CD of the ferret, we recently reported the presence of retrogradely labeled neurons in the DMV after 5 days, some of which were double labeled (16). The direct nature of these projections to the CD and LES was confirmed with the retrograde monosynaptic tracer, cholera toxin B (unpublished data). Control injections of the neuronal tracers into the abdominal space around the CD or LES failed to label cells in the DMV after a comparable time period, thus demonstrating that the projections to the CD were not due to leakage of the virus into the LES or the surrounding stomach tissue.

How DMV stimulation with L-glutamate results in changes in the frequency of CD bursts per minute is puzzling. We suggest that some DMV neurons are conditional pacemakers and that their excitation with L-glutamate could directly increase their rate of burst discharge or indirectly (via release of GABA from nerve terminals in the DMV) decrease their rate of burst discharge. There is evidence that a receptor for L-glutamate is present at GABAergic synapses and that its stimulation will facilitate GABA release (18). It should be noted that the DMV-to-CD pathway was not responsible for the EMG amplitude decrease observed with intermediate DMV stimulation due to the lack of effect of ipsilateral vagotomy on the response. Instead, we assume that the amplitude change was mediated via the phrenic motor nucleus.

Another new finding is drawn from data obtained with L-glutamate-induced excitation of intermediate DMV and the control of the duration of LES relaxation. That control involves the release of VIP at the level of the LES smooth muscle. Prolongation of the duration of LES relaxation was apparent when we tested the effect of intermediate DMV stimulation after pretreating ferrets with the NOS inhibitor L-NAME. Without NO release, LES relaxation (as reflected by the decrease in pressure) was longer. Conversely, without VIP acting at the LES smooth muscle the duration of LES relaxation was reduced.

Abrahams et al. (1) reported that L-glutamate microinjected into the intermediate and caudal areas of the DMV relaxed the LES, while L-glutamate microinjected into the rostral DMV contracted the LES. However, Abrahams et al. (1) did not test vagotomy on the LES contraction elicited from the rostral site. Hence, whether or not the response was mediated by the vagus was not addressed. In addition, they used bilateral vagotomy instead of ipsilateral vagotomy to determine the role of the vagus in the responses evoked from the intermediate and caudal areas of the DMV. The problem with bilateral vagotomy vs. ipsilateral vagotomy, as mentioned above, is that it will not distinguish between effects elicited from the DMV and from the NTS (8). Furthermore, no clear distinction was made between the mechanisms for LES relaxation evoked from the caudal area vs. the LES relaxation evoked from the intermediate area. Thus from their study, the profile of LES effects from both areas is similar. In our study, data obtained with L-NAME + VIP receptor antagonist separated the responses from the two DMV areas. LES relaxation produced from the intermediate DMV was mediated by both NO and VIP, while LES relaxation produced from the caudal DMV was not mediated by NO and VIP. Thus data from Abrahams et al. (1) suggest two separate populations of DMV neurons (caudal/intermediate population and rostral population), while our data suggest at least three separate populations (caudal, intermediate, and rostral populations).

Most of the published information on extrinsic neural control of the LES relates to the effects of electrical stimulation of peripheral autonomic nerves such as the cervical vagus nerve [e.g., Blackshaw, et al. (5)]. With the new information we now have about stimulating the cells of origin of these cervical vagal fibers, we conclude that stimulation of efferent fibers of the cervical vagus nerves should influence the LES in a way that would resemble the effects of simultaneous activation of all three divisions of the DMV. This appears to be the case based on results reported by Blackshaw et al. (5). Electrical stimulation of the peripheral cut end of either the right or left cervical vagus nerve of the ferret produced a triphasic response in LES pressure, namely, a brief decrease during stimulation, followed by a brief increase upon cessation of stimulation, and finally a prolonged decrease that followed the brief increase in pressure. The first phase of LES relaxation appeared to be mediated by NO release and based on the findings of the present study, is presumably due to activation of efferent vagal fibers originating from the intermediate division of the DMV. The s phase of LES contraction was blocked by atropine and may be due to activation of efferent vagal fibers originating from the rostral division of the DMV. The third phase (late inhibitory response) was not significantly altered by inhibiting NOS and therefore was considered not to be mediated by NO. This pharmacological profile of the third-phase response fits with our data obtained by stimulating the caudal division of the DMV. Data obtained with L-glutamate-induced activation of

<table>
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<tr>
<th>Area of DMV</th>
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<tr>
<td>CD Freq</td>
<td>CD Amp</td>
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<tr>
<td>Control</td>
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<td>Post-L-NAME+VIPA</td>
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Freq, frequency; Amp, amplitude; ↑, increase; ↓, decrease; ↔, no effect; NS, not studied; ▲, earlier onset, decreased duration; ★, onset delayed, duration increased; ●, reduced but not blocked.

Table 4. Summary of data from DMV stimulation studies
each of the three divisions of the DMV and electrical stimulation of the cervical vagus nerve would differ of course because the former would evoke selective changes in LES pressure, while the latter would evoke more complex changes in LES pressure. An interesting question posed by comparing DMV stimulation with cervical vagus stimulation is whether it (i.e., cervical vagus stimulation) would alter the EMG activity of the CD. When vagal stimulation was tested, no effect on CD was observed (2). This is consistent with our data in that EMG amplitude of the CD is not mediated by the efferent vagus nerve and that control of the frequency of EMG bursts of activity requires intervention at the level of the pacemaking neurons in the DMV.

One of the reasons for measuring fundus tone was to determine whether a site exists in the DMV that upon stimulation would simultaneously affect both the LES pressure and fundus tone. Since extensive coinervation of both the LES and fundus has been reported in the ferret (15), our expectation was that a positive result would be obtained. Indeed, excitation of all three areas of the DMV produced simultaneous changes in both LES pressure and fundus tone (Table 4). The other reason for measuring fundus tone in the ferret was to assess whether the DMV pathway that influences the fundus contains NO-releasing enteric neurons as proposed by others in the rat (14, 23, 40). Excitation of DMV neurons in the intermediate and rostral areas of the nucleus in the ferret only increased fundus tone, which was always prevented by ipsilateral vagotomy (indicative of DMV stimulation) and intravenous atropine methyl bromide (indicative of enteric neurons releasing acetylcholine) (Table 4). We did not observe any evidence for activation of a NANC pathway to the fundus with stimulation of the intermediate DMV even though the effects produced on the LES were always inhibitory through activation of a NANC pathway. These results obtained with strain-gauge recordings from the fundus of the ferret are similar to our recently reported findings in the rat (8) by using an intragastric balloon.

In the present study in the ferret, we did obtain evidence for activation of the NANC pathway to the fundus with stimulation of the caudal DMV. However, the decrease in fundus tone noted was not mediated by NO (or VIP), as blockade of nitric oxide synthesis and VIP receptors did not prevent this decrease. These results obtained in the ferret are similar to those obtained in the rat (8). In this species, stimulation of the caudal DMV results in a decrease in intragastric pressure, which is blocked by ipsilateral vagotomy but unaffected by intravenous 1-NAME. These results from the rat indicate that the functional inhibitory pathway arising from the caudal DMV to the gastric smooth muscle does not employ NO as a neurotransmitter. This is contrary to some studies (14, 21, 23, 40) that report of an inhibitory NANC pathway to the fundus from the intermediate DMV (34). Altogether, these observations suggest that a DMV-NANC pathway from the intermediate DMV is important for sphincter control but not for gastric smooth muscle control.

The larger question of why there are three separate pools of DMV neurons exerting control over the LES needs to be addressed. Recognition of these three populations may be important in trying to understand vagovagal reflex control of the LES, possible pathophysiological processes leading to sphincter disorders such as acid reflux and achalasia, and a new basis for developing drugs for therapy of these sphincter disorders.

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