Characterization of noradrenergic transmission at the dorsal motor nucleus of the vagus involved in reflex control of fundus tone

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Herman MA, Niedringhaus M, Alayan A, Verbalis JG, Sahibzada N, Gillis RA. Characterization of noradrenergic transmission at the dorsal motor nucleus of the vagus involved in reflex control of fundus tone. Am J Physiol Regul Integr Comp Physiol 294: R720–R729, 2008. First published January 16, 2008; doi:10.1152/ajpregu.00630.2007.—Quantitative analysis of innervation to dorsal motor nucleus of the vagus (DMV) fundus-projecting neurons indicates that ~17% of input neurons are noradrenergic. To determine whether this small percentage of neurons innervating DMV output to the stomach is physiologically relevant, we evaluated the role of norepinephrine at the DMV in mediating vagovagal reflex controlling the fundus. A strain gauge was sutured onto the fundus of isoflurane-anesthetized rats to monitor changes in tone evoked by esophageal distension (ED). ED produced a decrease in fundus tone of 0.31 ± 0.02 g (P < 0.05), which could be reproduced after a 30-min interval between distensions. Bilateral cervical vagotomy and/or pretreatment with intravenous atropine methylbromide prevented the reflex-induced fundus relaxation. In contrast, intravenous Nω-nitro-L-arginine methyl ester had no effect. Bilateral microinjection of α2-adrenoceptor antagonists (yohimbine and RS-79948) into the DMV also prevented the response. Before microinjection of α1-adrenoceptor antagonists, ED decreased fundus tone by 0.33 ± 0.05 g (P < 0.05). After agonist microinjection, ED decreased fundus tone by only 0.05 g (P < 0.05). Bilateral microinjection of prazosin into the DMV had no effect on the response. Microinjection of norepinephrine into the DMV mimicked the effect of ED and was also prevented by prior microinjection of an α2-adrenoceptor antagonist. Our results indicate that noradrenergic innervation of DMV fundus-projecting neurons is physiologically important and suggest that norepinephrine released at the DMV acts on α2-adrenoceptors to inhibit activity in a cholinergic-cholinergic excitatory pathway to the fundus.

gastric tone; norepinephrine; rat

OUR LABORATORY recently reported ultrastructural evidence for selective noradrenergic innervation of fundus-projecting neurons in the dorsal motor nucleus of the vagus (DMV) of the rat (22). Examination of synaptic contacts onto DMV fundus-projecting neurons that were identified by injection of β-subunit of cholera toxin conjugated to horseradish peroxidase (CTB-HRP), a retrograde neuronal tracer into the fundus, revealed that 17.4 ± 2.7% of the terminals showed dopamine-β-hydroxylase immunoreactivity (DBH-ir). In contrast, synaptic contacts onto DMV antrum-projecting neurons were devoid of DBH-ir terminals. These findings raised the question of whether noradrenergic afferent synaptic input to DMV fundus-projecting neurons that comprise only 17.4 ± 2.7% of the total synapses is physiologically important. To determine this, we employed a vagovagal reflex that controls fundus tone in the rat. This gastric relaxation reflex has been recently described by Rogers and colleagues (13, 24). An important aspect of this reflex is that it allows a change in fundus tone, as measured by a miniature strain gauge transducer, to be the experimental end point. The reflex is activated by distension of the thoracic esophagus with a fluid-filled balloon for 1 min, which produces consistent relaxation of the fundus.

The first purpose of our study was to utilize this “esophageal-gastric reflex” (EGR) (13) and determine whether blockade of norepinephrine receptors in the DMV via microinjection of adrenoceptor antagonists would prevent EGR-induced fundic relaxation. In performing these studies, we also sought to determine the identity of the adrenoceptor that mediates the reflex-induced synaptic transmission at the DMV. In an earlier study, Fukuda et al. (10) demonstrated that excitation of the nucleus tractus solitarii (NTS) noradrenergic pathway to the DMV in a rat brain slice preparation affected α2- but not α1-adrenoceptors on DMV neurons. However, both α2- and α1-adrenoceptors have been shown to be present on the same DMV neuron (10, 18). Hence, it is plausible that the α2-adrenoceptor is in the synapse, whereas the α1-adrenoceptor is extrasynaptic [data of Fukuda et al. (10)].

The second purpose of our study was to assess whether microinjection of norepinephrine into the DMV would mimic the effects of the EGR on fundus tone by activating the same adrenoceptor at the DMV. According to Rogers and colleagues (13, 24), the EGR that is evoked by esophageal distension with a 0.16-ml fluid-filled balloon is mediated by dual vagal innervation of the fundus, consisting of DMV pathways containing inhibitory nitrergic and excitatory cholinergic enteric neurons. Norepinephrine released at the DMV has been proposed by them to activate the inhibitory nitrergic pathway by excitation of α1-adrenoceptors and to inhibit the excitatory cholinergic pathway by activation of α2-adrenoceptors on DMV neurons (13, 24). The evidence for this proposal is indirect. It is based, in part, on data obtained with antagonists applied to the floor of the fourth ventricle (24) and, in part, on data obtained with tests of intravenously administered atropine methylnitrate and NGω-nitro-L-arginine methyl ester (L-NAME) on the EGR (13). To date, no data have been reported on the effect of microinjection of drugs that block the α1-adrenoceptors at the DMV on the EGR.

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A third purpose of our study was to repeat tests of intravenous quaternary atropine and l-NAME administration and observe their effect on the EGR. By pursuing this third purpose, our aim was to obtain data that would define the role of the dual inhibitory nitricergic and excitatory cholinergic pathways in noradrenergic-induced effects on DMV neurons.

**METHODS**

### Animals and Surgical Preparation

All experiments were performed on adult male Sprague-Dawley rats (250–350 g; Taconic, Rockville, MD) that were fasted overnight (16–24 h) with water available ad libitum. Animals were anesthetized with isoflurane administered via a nose cone (5% induction, 2.5% maintenance) vaporized with 95% oxygen and 5% CO₂. Adequate depth of anesthesia was monitored via toe pinch and corneal reflex for the duration of the experiment. Body temperature was maintained at 37 ± 1°C with an infrared heating lamp. After induction of anesthesia, the nose cone was switched to an intubation tube subsequent to tracheotomy. We chose isoflurane for our studies because of our recent success in using this anesthetic to study hindbrain control of upper gastrointestinal function in the ferret (20).

The carotid artery was cannulated with polyethylene tubing (PE-50) connected to a pressure transducer (sensitivity: 5 μV·mmHg⁻¹) for continuous monitoring of arterial blood pressure. Blood pressure was measured for the purpose of monitoring the physiological state of the animal during the course of the experiment. The pressure transducer was connected to a bridge amplifier and data acquisition system (PowerLab; AD Instruments, Colorado Springs, CO). The external jugular vein was cannulated with polyethylene tubing (PE-50) for intravenous administration of drugs. Both cerebral vagus nerves were carefully isolated and individually looped with a silk suture for selective sectioning during the course of the experiment.

The stomach and duodenum were then exposed via a midline abdominal incision. To measure changes in fundus tone, a miniature strain gauge transducer (2 × 4 mm; RBI Products), calibrated before each experiment, was then sutured to the fundus, oriented along the circular smooth muscle fibers. The strain gauge was coupled to a bridge amplifier and data acquisition system (PowerLab). To provide uniform preload conditions, a low-compliance balloon constructed from a latex condom was inserted through the pylorus via a small incision in the distal duodenum. The balloon was placed in the fundus and secured with ligatures around the duodenum. At the start of each experiment, the balloon was inflated with 2 ml of warm water to produce a mild and consistent preload strain (24) against which the fundus could relax. This preload strain was maintained for the duration of the experiment.

### Esophageal Distension Technique

The esophageal distension balloon was constructed similar to that described by Hermann et al. (13). It was fabricated from a silicone tubing (length 15 mm × inner diameter 0.5 mm × outer diameter 1 mm) that was attached to a noncompliant 5-F Fogarty occlusion catheter. The catheter was in turn connected to a 1-ml syringe. The balloon was orally inserted into the thoracic esophagus and positioned ~1 cm above the esophageal hiatus for the duration of the experiment. At the end of each experiment, the location of this esophageal distension balloon was reconfirmed visually. During distension, the balloon was inflated with 0.1 ml of water, increasing the outer diameter of the balloon to 4 mm. According to Ferreira et al. (9), a similar degree of distension (i.e., 0.16 ml) increases the outer diameter of the esophagus from ~2 to 4 mm. In all experiments, the balloon was distended for a period of 1 min.

All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research and with the approval of the Animal Care and Use Committee of Georgetown University, Washington, DC.

### Microinjection Procedure

All animals were placed in a small animal stereotaxic frame (Kopf Instruments, Tujunga, CA) in a prone position. Before stereotaxic surgery, all animals were administered dexamethasone (0.8 mg sc) to minimize swelling of the brain. A partial craniotomy was performed, the dura was reflected, and the cerebellum was partially retracted to expose the dorsal medulla. In all studies, the calamus scriptorius (CS) (i.e., the caudal tip of the area postrema) was used as a reference point for determining the coordinates for micropipette placement. The stereotaxic coordinates for the DMV were as follows: anteroposterior axis = 0.5 mm, mediolateral axis = 0.5 mm, and dorsoventral axis = 0.6 mm. The precise location of the DMV in our microinjection studies was functionally assessed using the L-glutamate microinjection method, which is described in detail in our earlier work (4, 8).

All microinjections were performed with double-barreled glass micropipettes (inner diameter 0.3 mm, tip diameter 30–60 μm; Frederick Haer, Bowdoinham, ME) that were inserted into the DMV at an angle 30° from perpendicular. Micropipettes were connected to polyethylene tubing (PE-50) that was in turn connected to a syringe for loading and unloading of drugs via positive or negative pressure, respectively. Drugs were microinjected within 5–10 s in 30-nl volumes determined by a calibration tape (Formaline 9006B; Wheeling, IL) affixed to the micropipette.

### Histological Verification of Microinjection Sites

Upon completion of each experiment, animals were euthanized with a lethal dose of pentobarbital sodium. The brain of each animal was rapidly removed and placed in a 4% buffered paraformaldehyde-20% sucrose solution for at least 24 h. After fixation/cryoprotection, brains were then cut on a cryostat into serial 50-μm coronal sections and mounted on slides. Subsequently, tissue was stained with neutral red, dehydrated, cleared, and coverslipped. The location of each microinjection site was identified, and a camera lucida drawing of it was made in relation to the nuclear groups as defined by the atlas of Paxinos and Watson (21).

### Experimental Design and Protocols

Fundus tone and blood pressure were monitored and recorded in all the experiments performed. In all studies, there was a minimum interval of 10 min between application of a gastric preload and the first esophageal distension. At least two reproducible EGR responses were obtained before the effects of experimental manipulations (e.g., vagotomy, agonist and antagonist pretreatments) on EGR responses were assessed. A 30-min interval between distensions was found to be sufficient to provide a consistent reproducible EGR response. At the end of each experiment, intravenous sodium nitroprusside (50 μg/kg) was routinely administered both to confirm the direction of the strain gauge transducer signal recorded and to determine that the stomach was capable of further relaxation following an experimental intervention (see Fig. 2B, inset).

**Bilateral cervical vagotomy.** After two reproducible EGR responses were obtained, the cervical vagus nerves were bilaterally severed. To obtain a stable baseline before repeating esophageal distension, an interval of at least 10 min postvagotomy was allowed.

**Intravenous l-NAME and atropine methylbromide.** l-NAME was administered as an intravenous bolus injection of 10 mg/kg. This dose was used in previous studies (13, 16, 27), including those conducted by Hermann et al. Focusing on the details of one of these studies [Takahashi and Owyang (27)], vagal pathways mediating the gastric accommodation reflex were characterized in rats (in vivo). Gastric distension using a 6-ml volume produced an increase of 9.0 ± 1.0 cmH₂O of intragastric pressure. After vagotomy, gastric distension was prevented.
produced an increase of 16.8 ± 1.9 cmH2O. These results were interpreted as indicating that the loss of vagus nerves interfered with the ability of the stomach to relax and to accommodate a volume of 6 ml. Pretreatment with a bolus intravenous injection of L-NAME (10 mg/kg) had a similar effect (intragastric pressure rose to 13.5 ± 1.6 cmH2O), which was nearly identical to that seen with hexamethonium pretreatment (13.9 ± 1.2 cmH2O). The effect of L-NAME on the pressure increase evoked by gastric distension was antagonized by p-adrenoreceptors.

To further justify the dose of L-NAME (10 mg/kg iv) in the present study, this dose of L-NAME was shown by us to be effective in blocking inhibitory nitricergic transmission at the lower esophageal sphincter (LES), since this dose altered the LES response to DMV stimulation (20).

In the present study, after two reproducible EGR responses were obtained, antagonists to nitric oxide synthase or muscarinic cholinergic receptors were administered. Esophageal distension was then repeated 5–7 min after L-NAME administration. The choice of a 5- to 7-min interval between L-NAME administration and esophageal distension was dictated by the need to obtain a stable baseline and to decrease the probability of significant central nervous system effects of L-NAME (17).

Atropine methylbromide was administered as an intravenous bolus injection of 0.1 mg/kg. This dose was chosen on the basis of its effective block of muscarinic receptors in our earlier study in the rat (4). Esophageal distension was then repeated 10 min after atropine methylbromide administration.

*Bilateral microinjection of α-adrenoreceptor antagonists into the DMV.* After two reproducible EGR responses were obtained, the micropipette was inserted into the DMV. l-glutamate evoked responses were then evaluated to confirm the placement of the micropipette tip in the DMV (4). For the α1-adrenoreceptor studies on the EGR, yohimbine hydrochloride (500 pmol/30 nl) or RS-79948 (100 pmol/30 nl) was bilaterally microinjected into the DMV. The dose of yohimbine was selected on the basis of our previous work (8). The dose of RS-79948 was initially determined on the basis of its relative potency compared with yohimbine (29) and then by further testing against two doses of norepinephrine microinjected into the DMV. Specifically, a dose of 100 pmol RS-79948 completely prevented 10 pmol of norepinephrine from decreasing fundus tone: 100 pmol of RS-79948 only partially counteracted a large dose of 100 pmol of norepinephrine microinjected into the DMV from decreasing the fundus tone. Since 100 pmol of RS-79948 was effective against 10 pmol of norepinephrine, we used this dose in the studies of EGR.

For the α1-adrenoreceptor studies, prazosin hydrochloride (100 pmol/30 nl) was bilaterally microinjected into the DMV. This dose of prazosin was determined on the basis of functional antagonism of the α1-adrenoreceptor selective agonist phenylephrine. Phenylephrine (100 pmol/30 nl) was microinjected into the medial subnucleus of the nucleus tractus solitarii (mNTS), where it has been reported to exert inhibitory effects on gastric motility (12). Two reproducible responses to phenylephrine were obtained with a 1-h interval between microinjections. In four rats, phenylephrine decreased fundus tone by 0.34 ± 0.03 g (P < 0.05). In an additional two rats, prazosin (100 pmol/30 nl) was microinjected following two phenylephrine microinjections. In these two rats, phenylephrine initially decreased fundus tone by 0.44 ± 0.3 g. Five minutes after prazosin microinjection, a repeat phenylephrine microinjection resulted in a +0.01 ± 0.00 g EGR response. This was the basis for using a prazosin dose of 100 pmol/30 nl in our study. After microinjection of α1-adrenoreceptor antagonists into the DMV, an interval of 5–10 min was used to establish a stable baseline before testing the EGR.

*Unilateral microinjection of norepinephrine into the DMV.* Procedures for microinjection into the DMV were the same as described for α-adrenoreceptor antagonists. Norepinephrine was unilaterally microinjected, and the effect on fundus tone was observed. Two doses of norepinephrine were studied. In studies characterizing the response to norepinephrine microinjection, a dose of 100 pmol/30 nl was used. After two reproducible responses to norepinephrine were obtained, both cervical vagus nerves were sectioned, and norepinephrine was again microinjected after an interval of 10 min. Studies characterizing the antagonism of norepinephrine microinjection by the α2-adrenoreceptor antagonist RS-79948 were carried out using a norepinephrine dose of 10 pmol/30 nl. Both doses of NE produced similar decreases in fundus tone. In these studies, l-glutamate was not used because our microinjection techniques utilized double-barreled pipettes. As such, studies of the interaction of two drugs, namely, norepinephrine and antagonists of norepinephrine, left no room for identifying the DMV with l-glutamate. Instead, we identified the DMV with norepinephrine, which we had learned would produce a characteristic decrease in fundus tone. Two reproducible responses to unilateral norepinephrine microinjection were obtained with an hour interval between microinjections. RS-79948 (100 pmol/30 nl) was then microinjected into the same site. Unilateral microinjection of norepinephrine (100 pmol/30 nl) was then repeated after an interval of 10 min.

**Drugs and Chemicals**

All drugs were purchased from Sigma (St. Louis, MO), with the exceptions of RS-79948 hydrochloride (Tocris, Ellisville, MO), sodium nitroprusside (Abbott Labs, Chicago, IL), isoflurane (Baxter, Deerfield, IL), and dexamethasone (Elkins-Sinn, Cherry Hill, NJ). All drugs were dissolved in 0.9% saline, and the solution was brought to a pH of 7.0–7.4, except yohimbine and prazosin, which were dissolved in double-distilled water, and the solution was brought to a pH of 6.0–6.5.

**Data Analysis**

The end point used to evaluate the EGR was the maximum change in fundus tone. Maximum change was determined by subtracting the gram tension at the point of maximum decrease in fundus tone from the average fundus tone over the 3-min interval preceding distension. In experiments where a phasic motility pattern was present, baseline values for tone were derived from averaged maximum decreases over 3 min preceding experimental manipulation. Values for maximum change during experimental manipulations were derived in a similar way. A graphical representation of this technique is shown in Fig. 1. The statistical significance of this response was determined using a one-sample t-test with a criterion for statistical significance of P < 0.05. In all cases, the EGR was performed twice before any experimental manipulation was carried out. The average value of both preexperimental manipulation responses was used to determine the control EGR value. Statistical significance between pre- and posttreatment EGR responses was determined using a paired t-test, and the criterion for statistical significance was set at P < 0.05. Comparison of norepinephrine microinjection responses before and after each experimental intervention was determined in the same way.

**RESULTS**

*Effect of Bilateral Cervical Vagotomy, Intravenous L-NAME, and Intravenous Atroline Methylbromide on the EGR*

In the first four animals studied, balloon inflation with 0.1 ml of water produced a relaxation of the fundus. The average relaxation response measured as a decrease in gram tension was 0.31 ± 0.02 g (P < 0.05). A repeat esophageal distension performed 30 min after the initial distension produced a nearly identical response (0.32 ± 0.05 g, P < 0.05; see Fig. 2B). This degree of fundus relaxation is slightly larger than that reported by Hermann et al. (13) using 0.16 ml to distend the esophageal balloon (see control responses in Fig. 2 of Ref. 13).

Our first goal before testing α1-adrenoreceptor antagonists in the DMV on the EGR was to determine whether this response...
was mediated by the vagus nerves. Thus, after obtaining two repeatable fundus relaxation responses, we performed bilateral cervical vagotomy and summarized the data in Fig. 2A. As can be noted, bilateral cervical vagotomy completely prevented the EGR. A representative experiment appears in Fig. 2B. Bilateral cervical vagotomy per se significantly increased baseline fundus tone by 0.28 ± 0.06 g (P < 0.05).

Our second goal was to obtain information that would help us decide which α-adrenoreceptor antagonist (α1 or α2) to test at the DMV to determine the role of noradrenergic synapses in this nucleus in mediating the EGR. Rogers and colleagues (13, 24) have concluded from their studies that DMV neurons containing α1-adrenoreceptors synapse in the stomach with nitric oxide-releasing enteric neurons. They have also concluded that DMV neurons containing α2-adrenoreceptors synapse in the stomach with acetylcholine-releasing enteric neurons. We reasoned that if intravenous L-NAME blocked the effect of esophageal gastric reflex (EGR) fundus tone, average minimum values of the points (y) during the EGR (horizontal bar) were subtracted from those points over a 3-min interval before distension (x). B: similarly, per se effects of experimental interventions (e.g., drug administration or bilateral vagotomy) were calculated from the average minimum points over a 3-min interval following baseline stabilization. Note: in the graph, experimental intervention (arrow) causes an increase in baseline. Both this and interventions that produced a decrease in baseline were similarly calculated.

Effect of Bilateral Microinjection of α2-Adrenoreceptor Antagonists Into the DMV on the EGR

Data obtained with intravenous L-NAME and atropine methylbromide suggested to us that relaxation of the fundus is due to activation of inhibitory postsynaptic α2-adrenoreceptors on DMV fundus-projecting neurons and not the result of activation of excitatory postsynaptic α1-adrenoreceptors on DMV fundus-projecting neurons. To test this, we employed two antagonists of α2-adrenoreceptors, namely, yohimbine and RS-79948, to see whether they could counteract the decrease in fundus tone produced by the EGR. As shown in Fig. 3A, bilateral microinjection of either yohimbine (500 pmol, n = 4) or RS-79948 (100 pmol, n = 2) into the DMV counteracted the EGR. Also shown in Fig. 3C is the degree of antagonism of the reflex when data from the studies of both α2-adrenoreceptor blockers are combined (n = 6). Using these combined data, control relaxation of the fundus with esophageal distension was 0.33 ± 0.05 g; P < 0.05. After bilateral microinjection of the α2-adrenoreceptor antagonists into the DMV, relaxation of the fundus was only 0.05 ± 0.06 g (P > 0.05). Hence, the magnitude of blockade with α2-adrenoreceptor antagonists was similar to the magnitude of blockade observed with either bilateral cervical vagotomy (Fig. 2A) or intravenous atropine methylbromide (Fig. 2C). Representative experiments performed with yohimbine and RS-79948 are shown in Fig. 3B. The microinjection sites for the yohimbine experiment shown in Fig. 3B appear in Fig. 3D. The microinjection sites for the six experiments summarized in Fig. 3C are shown in Fig. 4. α2-Adrenoreceptor blockade per se decreased baseline fundus tone by 0.15 ± 0.03 g (P < 0.05). Control experiments were performed investigating the effects of bilateral microinjection of vehicle for yohimbine into the DMV (double-distilled water brought to a pH of 6.0; n = 3). In these control studies, no effect was observed on the EGR (data not shown).

Effect of Unilateral Microinjection of Norepinephrine Into the DMV on Fundus Tone

If norepinephrine is the neurotransmitter at the DMV mediating esophageal distension-induced fundus relaxation, then norepinephrine microinjected into the DMV should mimic the α2-adrenoreceptor mediated relaxation of the fundus evoked by esophageal distension. To test this, we unilaterally microinjected two doses of norepinephrine into the DMV and noted changes in fundus tone. The first dose tested was 100 pmol, and
the data are summarized in Fig. 5A. As can be noted, norepinephrine unilaterally microinjected into the DMV of four rats produced a decrease in fundus tone. The magnitude of the decrease was similar to that observed with esophageal distension. In each of the four animals, ipsilateral vagotomy was performed after obtaining two reproducible responses with 100 pmol of norepinephrine. Ipsilateral vagotomy completely prevented norepinephrine-induced fundus relaxation (Fig. 5A). Figure 5B shows a representative tracing of this effect. The microinjection site for the experiment shown in Fig. 5B appears in Fig. 5C. Unilateral microinjection sites were confirmed for each experiment in this series (data not shown; available on request).

In another four animals, we tested a 10-pmol dose of norepinephrine at the DMV. This lower dose produced a similar decrease in fundus tone (Fig. 6A), and a representative experiment illustrating the time-action curve of its effect is shown in Fig. 6B. Ipsilateral vagotomy was not tested on the fundus relaxation produced by the lower dose of norepinephrine, because it was assumed that since ipsilateral vagotomy blocked the fundus relaxation produced by 100 pmol of norepinephrine, it would also block the fundus relaxation produced by 10 pmol of norepinephrine.

We did test the ability of RS-79948 (100 pmol) to block the fundus relaxation produced by 10 pmol of norepinephrine in four rats. After two reproducible responses were obtained with unilateral microinjection of norepinephrine, RS-79948 was microinjected into the same site. Repeat microinjection of 10 pmol of norepinephrine was performed and had no significant effect on fundus tone (Fig. 6, A and B). The microinjection site for the experiment shown in Fig. 6B appears in Fig. 6C. RS-79948 unilaterally microinjected into the DMV per se produced a significant reduction in fundus tone (0.15 ± 0.02 g; \( P < 0.05 \)). All microinjection sites were confirmed for each experiment in this series (data not shown; available on request).

Effect of Bilateral Microinjection of an \( \alpha_2 \)-Adrenoceptor Antagonist Into the DMV on the EGR

We tested the \( \alpha_2 \)-adrenoceptor antagonist prazosin (100 pmol) bilaterally microinjected into the DMV to determine whether this agent would influence the EGR. Four rats were tested, and the results are summarized in Fig. 7A. As can be noted, prazosin pretreatment did not significantly alter the reflex-induced fundus relaxation. A representative experiment is presented in Fig. 7B. The microinjection sites for the exper-
B. Prazosin per se did produce a significant decrease in fundus tone (0.28 ± 0.03 g; P < 0.05). All microinjection sites were confirmed for each experiment (data not shown; available on request). Control experiments were performed investigating the effects of bilateral microinjection of vehicle for prazosin into the DMV (double-distilled water brought to a pH of 6.0; n = 3). In these control studies, no effect was observed on the EGR (data not shown).

DISCUSSION

The purposes of our study were to 1) utilize the EGR described by Rogers and colleagues (13, 24) and determine whether blockade of ~17% of the synaptic contacts with DMV fundus-projecting neurons exerts a physiologically relevant effect, 2) assess whether microinjection of norepinephrine into the DMV would mimic the effects of the EGR, and 3) assess the role of dual inhibitory nitrergic and excitatory cholinergic transmission in noradrenergic reflex-induced effects on DMV fundus-projecting neurons.

We accomplished the first purpose by determining the effect of bilateral microinjection of α2-adrenoreceptor antagonists on the EGR. Two chemically different α2-adrenoreceptor antagonists were tested, namely, yohimbine and RS-79948, and both were found to prevent ~85% of the fundus relaxation evoked by esophageal distension. Hence, we conclude that blockade of ~17% of the synaptic contacts with DMV fundus-projecting neurons is physiologically relevant. Indeed, our data indicate that the ~17% of synaptic contacts that use norepinephrine as the chemical messenger carry almost all of the information necessary to carry out the EGR.

In performing these studies we were able to obtain definitive information as to the type of adrenoreceptor that is important for the activation of the EGR. Data derived from microinject-
ing yohimbine, RS-79948, and prazosin bilaterally into the DMV demonstrate the prominence of the $\beta_2$-adrenoreceptor in synaptic transmission associated with EGR. No significant role of $\alpha_1$-adrenoreceptors was observed in this vagovagal response. In addition, since $\alpha_2$-adrenoreceptor blockade inhibited the EGR, there was no reason to assess the role of $\beta_1$-adrenoreceptors at the DMV.

It is our opinion that norepinephrine is a neurotransmitter, not a neuromodulator, at the fundus-projecting DMV neurons that take part in the receptive relaxation reflex. This opinion is based on our observations that norepinephrine is contained in nerve terminals synapsing with DMV fundus-projecting neurons (22) and that esophageal distension-induced decrease in fundus tone is virtually blocked by microinjection of $\alpha_2$-adrenoreceptor antagonists in the DMV. Moreover, microinjection of norepinephrine into the DMV mimics the effect of esophageal distension, and this effect is similarly blocked by $\alpha_2$-adrenoreceptor antagonists.

Rogers et al. (24) evoked the EGR with a physiological stimulus similar to ours but chose to deliver their $\alpha_2$-adrenoreceptor antagonists by way of the floor of the fourth ventricle. In doing this, yohimbine reduced EGR-induced fundic relaxation to 56% of control and prazosin reduced EGR-induced fundic relaxation to 55% of control. However, a combination of the two blockers reduced the distension-induced response to 28% of control. This is in contrast with our present study in which blockade of $\alpha_2$-adrenoreceptors at the DMV abolished ~85% of the EGR response. In addition, blockade of $\alpha_1$-adrenoreceptors in this nucleus was without any significant effect on this response. These contrasting results are most likely reflective of the differences in the method of delivery of the adrenoreceptor antagonists. In choosing to deliver the antagonists via the floor of the fourth ventricle, Rogers et al. (24) surmised that it would “maximize the exposure of the DMV to the antagonist....” However, as they later reported (13), this method of delivery also has a direct impact on the area postrema and the solitary nucleus. Both these nuclei have $\alpha_2$-adrenoreceptors (1, 6), and in the case of the solitary nucleus, $\alpha_1$-adrenoreceptors as well (12). Therefore, it is conceivable that blocking these adrenoreceptors via the fourth ventricle produced the confounding effects on the EGR. Furthermore, the inability of yohimbine to produce a more complete block of the EGR may have been due to the method of delivery.

Fig. 5. A: histogram representation of averaged responses to norepinephrine (NE) microinjection before and after ipsilateral vagotomy. $^*P < 0.05$ ($n = 4$). B: representative experimental tracing depicting fundus tone during the 2 control unilateral NE microinjections into the DMV (left and middle) and after ipsilateral cervical vagotomy (right). Arrows above each trace indicate the times when NE microinjections occurred. C: photomicrograph of unilateral DMV microinjection site. The box depicts the pipette track, and the dashed ovals show the outline of both DMVs.

Fig. 6. A: histogram representation of averaged responses to NE microinjection before and after microinjection of RS-79948. $^*P < 0.05$ ($n = 4$). B: representative experimental tracing depicting fundus tone changes during 2 unilateral NE microinjections into the DMV (left and middle) and after unilateral microinjection of RS-79948 (right). Arrows above each trace indicate the times when NE microinjections occurred. As shown, bilateral microinjection of RS-79948 prevented NE from decreasing fundus tone. Note that RS-79948, per se, decreased baseline fundus tone. The antagonism observed with RS-79948 was not due to the decrease in fundus tone, because subsequent administration of SNP retained its robust effect (data not shown). C: photomicrograph of unilateral DMV microinjection site. The box depicts the pipette track, and the dashed ovals show the outline of both DMVs.
to insufficient diffusion of the drug to the DMV in a high enough concentration.

It should be noted that α₂-adrenoceptors exist not only on postsynaptic membranes but also on axon terminals of the neuron that releases norepinephrine (i.e., autoreceptors) (3). Therefore, it is conceivable that norepinephrine synaptically released at the DMV could interact with presynaptic α₂-adrenoceptors to inhibit neural release of norepinephrine. Hence, administering α₂-adrenoceptor antagonists in our study would increase the amount of norepinephrine released during the EGR, thereby augmenting fundus relaxation. However, this was not the case, given that the EGR response was blocked. Thus we conclude that blockade of presynaptic α₂-autoreceptors did not contribute to the results of our study.

Another point for consideration is that not all of the afferent input to DMV gastric-projecting neurons synapse within the DMV. Several investigators have reported the presence of DMV dendrites that extend into the NTS, primarily into the subnucleus gelatinosus, just rostral to the obex [see Rinaman et al. (23)]. Ultrastructural analysis of these dendrites shows that they form synaptic contacts with vagal afferents in the NTS (23). It is therefore possible that α₁-adrenoceptors are present on these NTS projecting DMV dendrites. Hence, our lack of effect with prazosin might be explained by its microinjection into the DMV and not into the NTS.

Fig. 7. A: histogram representation of averaged control responses of the EGR compared with averaged EGR responses following bilateral prazosin microinjection (n = 4). Praz, prazosin. B: representative experimental tracing depicting fundus tone changes during EGR before (left) and after (right) bilateral prazosin microinjection into the DMV. Horizontal bars above each tracing indicate the time period of esophageal distension. C: photomicrograph of bilateral DMV microinjection sites. The boxes depict the pipette tracks, and the dashed ovals show the outline of both DMVs.

Focusing on the second purpose of our study, namely, whether microinjection of norepinephrine into the DMV mimics the effects of the EGR, our data indicate that it does in all respects. Norepinephrine microinjected unilaterally into the DMV reduced fundus tone that was prevented by ipsilateral vagotomy. This indicates that the drug, before ipsilateral vagotomy, was acting on DMV and not on NTS neurons (4). Similarly, blockade of the α₂-adrenoceptor significantly attenuated the effect of norepinephrine microinjection into the DMV on fundus tone. Thus fundus relaxation evoked by either EGR or norepinephrine is largely prevented by blockade of α₂-adrenoceptors. It should be noted that the lack of a significant degree of fundus relaxation produced by norepinephrine after α₂-adrenoceptor blockade in the DMV precluded testing of an α₁-adrenoceptor antagonist at this site.

Our third purpose was to assess the contribution of dual inhibitory nitricergic and excitatory cholinergic transmission in the EGR response. Our data demonstrate that it is the noradrenergic-induced inhibition of excitatory cholinergic transmission that is responsible for the EGR. This is based on our observations that 1) bilateral microinjection of α₂-adrenoceptor antagonists but not an α₁-adrenoceptor antagonist largely prevents the EGR; 2) norepinephrine microinjected into the DMV mimics the effect of the EGR, and does so by activating an α₂-adrenoceptor; and 3) intravenous atripine methylbromide but not intravenous L-NAME blocks the EGR.

We find ourselves in a situation similar to that of Hermann et al. (13), trying to reconcile differing results from two independent laboratories. Hermann and colleagues attempted to resolve their findings for evidence that two pathways are involved in this reflex (EGR) with our earlier finding (7) that the EGR is determined exclusively by the withdrawal of cholinergic tone induced by α₂-adrenoceptor-mediated inhibition of DMV neurons. In attempting to resolve these disparate findings, they raised the following criticisms vis-à-vis our studies: 1) we did not employ the same reflex-stimulating techniques they used and were probably studying a different vago-vagal reflex, namely, a gastro-gastric reflex [which we refuted; see Ferreira et al. (9)]; 2) we used an esophageal balloon distension measuring 10 mm in diameter, whereas their esophageal balloon distension measured ~4 mm in diameter; 3) we used an esophageal balloon stimulation volume of 0.7 ml, whereas theirs was 0.16 ml; 4) we used an intragastric balloon for measuring the end point of gastric motility, whereas they used a gastric wall strain gauge (applied to the fundus); and 5) we used α-chloralose as an adjunct to urethane anesthesia, which they report as inducing “adynamic ileus.” They used Inactin (thiobutabarbital) for anesthesia in their two studies (13, 24).

The question is whether these same criticisms can be leveled against the disparate findings of the present study. The answer is no, since the design of the present study addresses all five criticisms. We used a reflex-stimulating technique very similar to that used by Rogers and colleagues (13, 24). Indeed, our distension volume was less than that used by this research group (0.10 vs. 0.16 ml), although our end point of fundus relaxation was larger (0.34 g) than that reported by them (0.22 g; see Fig. 2 in Ref. 13). The diameter of our esophageal balloon (4 mm) when distended was in the range of that described by Hermann et al. (9, 13). Similarly, to record fundus tone, we used a miniature strain gauge applied to the...
fundus. Finally, we used isoflurane anesthesia in place of the α-chloralose-urethane cocktail that we employed in our earlier work (7).

Why are our results different from those of Rogers and colleagues? There are several possible explanations. One is the issue of anesthetics used. We employed isoflurane in our current study, whereas they employed a relatively high dose of Inactin (150–200 mg/kg ip). The usual dose of this anesthetic is 80–125 mg/kg ip (2, 5, 14, 15, 25, 26, 28, 30).

A further possibility involves the use of l-NAME. In administering this inhibitor of nitric oxide synthase (10 mg/kg iv), Hermann et al. (13) reported that it reduced the amplitude of fundus relaxation produced by esophageal distension to 30–70% of the original EGR response. The interval between l-NAME administration and a test of the EGR was 15 min (intravenous drug treatment preceded the gastric preload by 10 min, and gastric preload required 5 min to establish, for a total of 15 min). Hermann et al. (13) raised the possibility that the l-NAME could be acting centrally but discount this by stating that “previous work has shown that the central neuropharmacological components of the EGR circuit [does] not utilize nitrergic or cholinergic transmission,” citing four references. They failed to cite two references showing that nitrergic transmission at the NTS is involved in mediating a vagovagal reflex that produces inhibition of gastric motility (7, 8). Another point to consider is the earlier findings of Ma et al. (17) on the effect of intravenous administration of l-NAME (10 mg/kg) on medial NTS neurons in the rat. They showed that in 12 of 14 neurons recorded, l-NAME decreased their activity, an effect that was maximal 12–15 min after its administration. Hence, l-NAME does affect hindbrain neural activity in one of the two major hindbrain nuclei comprising the vagovagal reflex. In our study, the interval between l-NAME administration and the EGR test was 5–7 min. At this time interval, l-NAME appeared to have exerted a maximal effect as confirmed by the consistent rise in blood pressure. However, it was ineffective in blocking the EGR response.

The differences in findings between our study and the findings of Hermann et al. (13) may also be due to their dose of atropine methyl nitrate. The dose they used (50 μg/kg iv) reduced the amplitude of gastric relaxation to 52.0 ± 4.4% of control. The rationale given for using the atropine methyl nitrate dose is based on two cited references in their article. One of these, Takahashi and Owyang (27), used a similar but not identical dosing regimen (50 μg/kg bolus and continuous infusion of 20 μg·kg⁻¹·h⁻¹) and found that atropine had no effect on the gastric accommodation reflex in their study. Hence, the data of Takahashi and Owyang cannot be used as a source for indicating that 50 μg/kg atropine is a full muscarinic receptor blocking dose, because these receptors are not involved in mediating the accommodation reflex. The other citation is to P. Millard’s chapter in a textbook on clinical veterinary nursing (19). Millard lists the dose range of atropine (0.02–0.05 mg/kg) that is found in the contents of an anesthetic emergency kit. However, no reference is given for how the atropine dose was determined and/or whether the dose range applied to treating an anesthetic emergency in rats. We used a dose of atropine methylbromide (i.e., 0.1 mg/kg iv) that was twice as high as that used by Hermann et al. (13). In a previous study, this dose was effective in preventing l-glutamate microinjection at the DMV from increasing intragastric pressure (4). In the present study, we found that administration of atropine methylbromide (0.1 mg/kg iv) prevented ∼85% of the EGR response.

Reflecting on these latter two differences, namely, the time to peak effect of l-NAME on the EGR response and the atropine dose, we suggest that the different findings of Hermann et al. (13) may be due to a combination of an inadequate atropine dose and an NTS effect of l-NAME.

**Perspectives and Significance**

We employed the esophageal-gastric reflex described by Rogers and colleagues (24) as our test system for examining the role of noradrenergic transmission at the DMV in controlling fundus tone. We presume that the components of this reflex consist of afferent vagal neurons with sensory elements in the thoracic esophagus, second-order noradrenergic neurons originating in the NTS (22), and efferent vagal neurons projecting to the fundus. This reflex, commonly known as the receptive relaxation reflex (11), is partly responsible for the maintenance of an unchanging intragastric pressure after eating. Swallowing of food stretches the esophagus and reflexively (by receptive relaxation) reduces fundus tone, thus keeping intragastric pressure stable. Data from the present study suggest that this reflex operates by utilizing <20% of synaptic input to the fundus-projecting DMV neurons. Norepinephrine, acting at the NTS-DMV synaptic interface, inhibits ongoing activity of DMV motor neurons, leading to the EGR reflex. This effect is mediated entirely by α2-adrenoceptors; α1-adrenoceptors do not play a role. Our current data also show that the efferent vagal pathway involved in the receptive relaxation reflex is composed solely of cholinergic-cholinergic neurons. Enteric nitrergic neurons responding to preganglionic release of ACh do not contribute to this reflex.

In perspective, the view that central noradrenergic neurons “determine the brain’s global orientation concerning events in the external world and within the viscera” (3) is applicable to the vagovagal pathway that we studied. In this pathway, brain stem noradrenergic neurons respond to mechanical stimuli within the esophagus that in turn cause the DMV fundus-projecting neurons to inhibit the fundus, thus making room for ingested food without a rise in intragastric pressure.

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