Hindbrain chemical mediators of reflex-induced inhibition of gastric tone produced by esophageal distension and intravenous nicotine

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Ferreira, Manuel, Jr., Niaz Sahibzada, Min Shi, Mark Niedringhaus, Matthew R. Wester, Allison R. Jones, Joseph G. Verbalis, and Richard A. Gillis. Hindbrain chemical mediators of reflex-induced inhibition of gastric tone produced by esophageal distension and intravenous nicotine. Am J Physiol Regul Integr Comp Physiol 289: R1482–R1495, 2005. First published July 28, 2005; doi:10.1152/ajpregu.00003.2005.—The purpose of this study was to activate a vagovagal reflex by using esophageal distension and nicotine and test whether hindbrain nitric oxide and norepinephrine are involved in this reflex function. We used double-labeling immunocytochemical methods to determine whether esophageal distension (and nicotine) activates c-Fos expression in nitrergic and noradrenergic neurons in the nucleus tractus solitarii (NTS). We also studied c-Fos expression in the dorsal motor nucleus of the vagus (DMV) neurons projecting to the periphery. Esophageal distension caused 19.7 ± 2.3% of the noradrenergic NTS neurons located 0.60 mm rostral to the calamus scriptorius (CS) to be activated but had little effect on c-Fos in DMV neurons. Intravenous administration of nicotine caused 19.7 ± 4.2% of the noradrenergic NTS neurons 0.90 mm rostral to CS to be activated and, as reported previously, had no effect on c-Fos expression in DMV neurons. To determine whether norepinephrine and nitric oxide were central mediators of esophageal distension-induced decrease in intragastric pressure (balloon recording), Nω-nitro-L-arginine methyl ester microinjected into the NTS (n = 5), but not into the DMV, blocked the vagovagal reflex. Conversely, α2-adrenergic blockers microinjected into the DMV (n = 7), but not into the NTS, blocked the vagovagal reflex. These data, in combination with our earlier pharmacological microinjection data with nicotine, indicate that both esophageal distension and nicotine produce nitric oxide in the NTS, which then activates noradrenergic neurons that terminate on and inhibit DMV neurons.

vagus nerves; dorsal motor nucleus of the vagus; medial subnucleus of the tractus solitarii; α2-adrenergoreceptor; esophageal distension; c-Fos; nitric oxide synthase; central nervous system

THE MAIN NEURAL COMPONENTS of a vagovagal reflex consist of vagal afferent nerves (first-order neurons) that innervate second-order neurons of the nucleus of the solitary tract (NTS), which in turn synapse onto efferent vagal neurons in the dorsal motor nucleus of the vagus (DMV) (38). The efferent vagal neurons are considered to comprise two pathways, namely, a cholinergic excitatory pathway (i.e., a preganglionic vagal neuron synapsing onto a postganglionic cholinergic neuron) and a nonadrenergic-noncholinergic (NANC) inhibitory pathway (i.e., a preganglionic vagal neuron synapsing onto a NANC neuron) (6, 18, 37). Studies of the vagovagal reflex indicate that both efferent vagal pathways are influenced by sensory stimuli (1, 3, 8, 6, 20, 21, 35, 39). Thus afferent vagal activity could inhibit the stomach by simultaneous excitation of the DMV NANC pathway and inhibition of the cholinergic excitatory pathway (35).

The principal second-order neurons of the NTS that engage the third-order DMV neurons have been thought to be GABAAergic and glutamatergic (7, 9, 18, 52, 53, 58). In these reports, noradrenergic neurons are never considered as candidates for second-order neurons in the vagovagal reflex pathways. However, this is surprising for several reasons. First, Rea et al. (34) have shown that the DMV exhibits more noradrenergic terminals than any other major nucleus in the medulla oblongata. The origin of these terminals presumably arises from several noradrenergic cell groups, namely, A1, A2, A5, and A6 (23, 41, 46, 48, 56). Second, Sumal et al. (44) report that A2 noradrenergic neurons within the NTS are innervated by terminals from first-order vagal neurons in the nodose ganglion. Third, vagal afferent nerve stimulation has been described as affecting the firing rate of A2 neurons (26). Fourth, electrical stimulation of the A2 noradrenergic cell group evokes inhibitory postsynaptic potentials (IPSPs) in the DMV, which are blocked by the α2-adrenergoreceptor antagonist yohimbine (14). This also has been shown to be the case with some spontaneously recorded IPSPs at the DMV (14).

Our interest in the role of noradrenergic neurons in a vagovagal reflex was prompted by results obtained with intravenously administered nicotine showing that this substance also activates a vagovagal reflex, resulting in decreases in intragastric pressure (IGP) and fundus tone in the rat (12). Once we realized that nicotine exerted this effect, we employed this alkaloid as a tool to learn what central nervous system (CNS) neurotransmitters mediated the reflex-induced decreases in IGP and fundus tone. We assumed that nicotine was acting to excite a second-order inhibitory neuron and that the inhibitory neurotransmitter released was either GABA (18, 51, 53) or norepinephrine (14, 26, 34, 44). Blockade of the GABA A receptor at the DMV did not alter nicotine-induced decreases in IGP. However, blockade of the α2-adrenergoreceptor at the DMV did abolish most of the response (12). Our study also revealed that for this vagovagal reflex to operate, nitric oxide was required to act as a signaling molecule in the NTS. Our data indicated that the efferent projecting pathway out of the DMV affected by nicotine appeared to be only the cholinergic excitatory pathway. Evidence for this was a lack...
of any direct excitation by nicotine of peripherally projecting DMV neurons, based on c-Fos expression data (12).

Subsequent to our study showing that norepinephrine and nitric oxide are involved in synaptic signaling in gastrointestinal vagovagal reflex pathway and that NANC neurons are not involved, Rogers et al. (39) published results at odds with our findings. These investigators used esophageal distension to activate a vagovagal reflex and produce fundic relaxation in the rat. Although esophageal distension increased c-Fos expression in NTS neurons immunoreactive for tyrosine hydroxylase (TH), blockade of \( \alpha_2 \)-adrenoreceptors in the hindbrain only partially antagonized the reflex-induced fundic relaxation. To produce a blockade equivalent to that which we observed using yohimbine, they required a combination of yohimbine and the \( \alpha_1 \)-adrenoreceptor antagonist prazosin. Furthermore, suppression of nitric oxide formation in the hindbrain failed to influence this vagovagal reflex. Finally, in contrast to our findings, the NANC pathway was concluded to play a major role in mediating their vagovagal reflex.

The specific purpose of the present study was to employ the same reflex-stimulating technique as Rogers et al. (39), namely, esophageal distension, and to test whether previous results obtained with nicotine also hold true for esophageal distension.

**METHODS**

**Animals and Surgical Preparation**

Experiments were performed on male Sprague-Dawley rats (\( n = 77 \)) weighing 275–400 g (Taconic, Germantown, NY) in accordance with the National Institutes of Health guidelines for use of animals in research and with the approval of the Georgetown University Animal Care and Use Committee.

Before all experiments, with the exception of the c-Fos immunohistochemistry experiments, food was withheld overnight, whereas water was provided ad libitum. Animals were anesthetized with an intraperitoneal injection of a mixture (3 ml/kg) containing urethane (800 mg/kg) and \( \alpha \)-chloralose (60 mg/kg) dissolved in 3 ml of 0.9% saline. Body temperature was monitored with a rectal thermometer and maintained at \( 37 \pm 1^\circ \)C with an infrared heating lamp. To minimize brain swelling, we pretreated all animals that underwent neurosurgery with dexamethasone (0.8 mg sc).

Rats were intubated via the trachea to maintain an open airway and to institute artificial respiration when necessary. The carotid artery and the jugular vein were also cannulated with polyethylene tubing (PE-50) for monitoring blood pressure and for systemic infusion of drugs, respectively. Blood pressure was monitored with a pressure transducer that was coupled to a PowerLab data acquisition system (AD Instruments, Colorado Springs, CO).

To monitor intraluminal gastric pressure, an intragastric balloon (made from the little finger of a small latex glove, connected to polyethylene tubing, PE-160) was inserted into the stomach via the fundus and positioned around the corpus/antrum areas. The balloon was inflated (with warm saline, 2–3 ml) to produce a baseline pressure of 6–15 mmHg. This tubing was also connected to a pressure transducer, whose signal was channeled via a quad-bridge amplifier (AD Instruments) onto the PowerLab data acquisition system connected to a G4 Macintosh computer (Apple). Data obtained were analyzed as the decrease in IGP (mmHg) from the baseline value obtained over a 3-min period of time before drug or vehicle microinjection.

For brain microinjection experiments, to gain access to the dorsal medulla, the rats were positioned in a stereotaxic apparatus (David Kopf, Tujunga, CA). A partial dorsal craniotomy was performed to expose the medulla. After retraction of the cerebellum, the underlying dura and pia were cut and reflected. The caudal tip of the area postrema, the calamus scriptorius (CS), was viewed as a reference point for determining the microinjection coordinates.

**Brain Microinjection Technique and Histological Verification of Microinjection Sites**

Drugs were infused via a double-barrel pipette with an overall tip diameter of 30–60 \( \mu \)m. All microinjections were given bilaterally into either the medial subnucleus of the tractus solitarii (mNTS) or the DMV. Microinjections were administered within 5–10 s in a volume of 60 nl via hand-controlled pressure. Stereotaxic coordinates for injection (pipette was angled at 30° from the perpendicular) into the mNTS were 0.3–0.5 mm rostral to CS, 0.5–0.7 mm lateral to the midline, and 0.4–0.6 mm ventral to the dorsal surface of the medulla. Coordinates for the DMV were 0.3–0.5 mm rostral to CS, 0.3–0.5 mm lateral to the midline, and 0.5–0.7 mm ventral to the dorsal surface of the medulla. These coordinates are similar to those reported in an earlier study (12). In these earlier studies in our laboratory (12, 13), we demonstrated our ability to place microinjected drug into either the NTS or the DMV.

At the end of each experiment, the rat 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-\( \mu \)m-thick coronal sections and stained with neutral red. The location of the microinjection sites was studied in relation to nuclear groups by using the atlas of Paxinos and Watson (32). Initially, the gross micropipette track was located. The track was then followed under both dark-field and light-field optics to its deepest point and plotted. In most cases, our ~50-\( \mu \)m pipette tip produced a distinct track that could be clearly identified. In rare cases, when the pipette tip could not be located with confidence, data from those experiments were discarded. Camera lucida drawings were performed for each experiment to document all microinjection sites. It must be noted that although our micropipette produced tissue damage along its track, stable responses to L-glutamate microinjection could be elicited that were reproducible.

**Esophageal Distension Technique**

The description of the following technique applies to all experiments except those involving c-Fos immunohistochemistry. A Fogarty 5-F balloon catheter (Edwards LifeSciences, Irvine, CA) was used to produce stretch of the esophagus. The catheter was placed orally in the thoracic esophagus ~1 cm above the esophageal hiatus, as described by Rogers et al. (39). Its location was confirmed by direct visualization at the termination of the experiment. The effects of esophageal distension on gastric tone were tested by distending the balloon with fluid for a duration ranging from 15 to 60 s. In any given experiment, the duration of stretch was kept constant throughout the entire study. For most of the pharmacological microinjection studies, the volume used ranged from 0.6 to 0.8 ml.

**c-Fos Immunohistochemistry**

For c-Fos studies, male Sprague-Dawley rats, 275–400 g, were housed three to a cage in a temperature-controlled room with a regular light cycle. All rats were allowed to acclimate to the facility for at least 5–7 days on standard rat chow and tap water before experimentation. On the day of study, rats were denied access to food and water from 8:00 AM. Included in this study are brains from rats of a previous study in which intravenous doses of 56.5, 113, and 226 nmol/kg nicotine were administered (12). The purpose for including these brain tissues was for comparing data obtained by activating vagovagal reflexes with nicotine with data obtained by activating vagovagal reflexes with esophageal distension.

For c-Fos studies to be optimal, studies need to be performed in conscious animals, because most anesthetics that have been tested...
induce c-Fos expression (47). This was possible for our intravenous nicotine experiments (12), but anesthesia was required for investigations of the effects of esophageal stretch, because the stress involved with stretching the thoracic esophagus in conscious animals was assumed to be too great. In preliminary studies, we sought an anesthetic that would not cause a high baseline level of c-Fos activity. This was achieved by administering pentobarbital (70 mg/kg ip) to three rats and perfusing their brains 1 h later for c-Fos immunoreactivity. Three conscious animals were given intraperitoneal vehicle injection and euthanized 1 h later with an overdose of pentobarbital. On visual inspection of c-Fos immunoreactivity in brain stem sections, no apparent difference between the pentobarbital-anesthetized group and the conscious group was noted. On the basis of these data from our preliminary studies, we used pentobarbital-anesthetized rats for testing esophageal distension on c-Fos expression in the hindbrain.

After rats were anesthetized with 70 mg/kg ip pentobarbital, the balloon catheter was inserted orally and placed in the esophagus so that the tip was located ~1 cm above the esophageal hiatus in the thoracic esophagus. The balloon was then distended with fluid to produce a balloon diameter of 1.0 cm (0.7-ml volume). Distension duration was for 1 min.

In these experiments, two different protocols were used to distend the thoracic esophagus. One protocol involved distending the thoracic esophagus for 1 min and removing the brain for c-Fos analysis 60 min later. A control consisted of placing the catheter into the thoracic esophagus for 1 min but not inflating the balloon to distend the esophagus, followed by removal of the brain 60 min later for c-Fos analysis. The other protocol involved distending the thoracic esophagus for three 1-min periods. Each 1-min period of distension was separated by a 3-min rest period. Sixty minutes after this 12-min “test” protocol, brains were harvested and examined for the presence of c-Fos. A control consisted of placing the catheter into the thoracic esophagus for 12 min but not inflating the balloon to distend the esophagus. The brain of each animal was removed 60 min later for c-Fos analysis. Data for both protocols were similar and thus were pooled. Visual inspection of the dissected esophagus of control and test animals at the end of the experiment did not reveal any observable injury to the esophagus from either a 1-min-duration balloon distension or a 1-min-duration balloon distension applied three times separated by a 3-min rest period. By using a similar version of the first protocol for drug microinjection studies, relaxation of the fundus could be reproduced by repeated esophageal distension consistent with no injury to the esophagus.

In all of the above studies, 5 days before euthanasia, animals were given intraperitoneal injections of 0.8 mg of Fluoro-Gold (FG; Fluorochrome, Denver, CO) to retrogradely label DMV neurons projecting to the periphery. Of the 12 animals receiving FG, the DMV was analyzed for c-Fos expression in five rats undergoing esophageal distension and three rats serving as controls.

To remove the brain for c-Fos analysis (and for phenotyping neurons, see below), the thoracic cavity was opened, the inferior vena cava was clamped, and an 18-gauge gavage needle was inserted into the apex of the heart and routed to the entrance of the ascending aorta. Five hundred units of heparin were injected into the heart, and the right atrium was punctured to allow drainage. The animal was then perfused transcardially using methods described previously (42). The brains were post-fixed overnight in phosphate-buffered 4% paraformaldehyde and then stored in 25% sucrose until sectioned. Brain stems were cut into sequential 25-μm coronal sections with a freezing stage microtome (Jung Histoslide 2000; Deerfield, IL). The sections were collected into serially ordered sets through the rostralcaudal extent of the DMV so that each set contained 1:6 series of hindbrain sections spaced ~150 μm apart. The sections were stored at ~20°C in tissue culture dishes containing cryoprotectant (55) until they were processed.

To ensure that the immunohistochemical analyses were representative of the entire extent of the sectioned brain area, each analysis consisted of sections that were cut 150 μm apart (every 6th section). The tissue was processed using methods described previously (42). To identify FG-containing neurons, the same sections were double-stained with an antibody directed against FG (Chemicon, Temecula, CA), diluted 1:70,000 in PBS-Triton X-100. Peroxidase was attached to the antibody as described previously (42), and the presence of peroxidase was detected by incubating with diaminobenzidine and hydrogen peroxide in 0.05 M Tris-buffered NaCl (pH 7.2, 0.15 M). This reaction product was light brown. Throughout the staining procedure, the tissue was rinsed with PBS multiple times after each incubation step. The tissue was mounted on Superfrost Plus slides (Fisher Scientific), air-dried overnight, serially dehydrated in alcohol, cleared in Histoclear, and coverslipped with Histomount (National Diagnostics, Atlanta, GA).

Tissue slices were visualized using a Nikon Eclipse E600 microscope fitted with a linear encoder (type MSA 001-6; RSF Electronics, Rancho Cordova, CA) connected to a digital readout device (Microcode II; Boeckler Instruments, Tucson, AZ), a video camera (DEI-750; Optronics Engineering, Goleta, CA), and a microcomputer running the Bioquant software package (Bioquant Image Analysis, Nashville, TN). Using ×10 and ×20 objective lenses, the brain regions of interest (NTS and DMV) were outlined by using the brain atlas of Paxinos and Watson (32) as a guide. The numbers of total c-Fos-positive (+) cells in the NTS and the numbers of c-Fos (+) and FG (+) immunoreactive cells in the DMV were counted separately on each section. With the Bioquant software package, each individual immunoreactive cell was marked during the counting process, eliminating the possibility of double counting identified cells. For each animal, all single- and double-labeled neurons were counted from sections that were 1.05 mm rostral and 0.60 and/or 0.90 mm caudal to CS. The total number of positive cells in the area counted was then divided by the number of sections counted, and the result was expressed as c-Fos (+) (NTS) or c-Fos (+) and FG (+) (DMV) neurons per section.

**TH and Nitric Oxide Synthase Immunocytochemistry**

Alternate sets of c-Fos-labeled sections were further processed for the immunocytochemical localization of TH or nitric oxide synthase (NOS). Sections were rinsed thoroughly in PBS and then incubated for 48 h at 4°C in one of the following antisera: monoclonal mouse anti-TH (Chemicon; 1:50,000) or polyclonal rabbit anti-NOS antibody (Chemicon; 1:50,000). After incubation, sections were rinsed and incubated in the appropriate biotinylated secondary antisera (anti-rabbit or anti-mouse IgG; Vector) and rinsed in PBS. Sections were then processed as described above, using Vectastain reagents. Cytoplasmic TH or NOS immunoreactivity was detected with intensified diaminobenzidine. Tissue sections were then rinsed thoroughly in PBS, mounted on glass slides, air-dried, dehydrated through graded alcohols, cleared in Histoclear, and coverslipped with Histomount (National Diagnostics).

**Cytoarchitectural and Quantitative Analysis**

The Bioquant Tracing System was used to map the locations of immunolabeled cells in tissue sections. First, the distribution of c-Fos (+) cells was plotted in 12 coronal sections (spaced ~150 μm apart) through the medulla, beginning at the caudal NTS and extending through the rostral DMV. With a ×10 objective, the locations of all visible blue-black c-Fos immunoreactivity profiles were plotted, regardless of labeling intensity. For these plots and for quantitative analysis (see below), TH-immunoreactive (TH-IR) neurons were considered c-Fos (+) if they displayed a blue-black nucleus. Neurons were considered c-Fos negative (−) if they displayed either no nucleus or a nucleus free of blue-black immunoreactivity. The criteria used for plotting (and counting) a neuron as TH-IR or NOS-IR included the presence of brown cytoplasmic immunolabeling, even if the neuronal nucleus was not visible.
The TH(+) and NOs(+) neurons in the NTS were counted, and the percentage of these neurons expressing nuclear c-Fos immunoreactivity was determined. In each case, immunoreactive cells were counted in 12–14 sections from each set of double-labeled material, corresponding to the rostrocaudal levels used for mapping (see above).

Experimental Protocols

Before testing our hypothesis, namely, that CNS NO and CNS noradrenergic neurons are important components of a gastrointestinal vagovagal reflex, it was necessary to establish that esophageal distension-evoked reflex-induced changes involved only efferent vagal excitatory pathways to the stomach (with no sympathetic nervous system component) and also involved the hindbrain. To assess whether the esophageal distension reflex involved the hindbrain, we performed bilateral microinjection of tetrodotoxin (TTX) into the mNTS. Details about these protocols are listed below.

Bilateral cervical vagotomy (n = 6 animals). First, two control responses to esophageal distension were obtained. To ensure reproducible reflex-induced responses, we used a 25- to 30-min interval between reflex tests for this and the following protocols. Acute denervation of the parasympathetic nervous system was performed by exposing and isolating the vagus nerves in the cervical region and sectioning these nerves bilaterally. At least 10 min were allowed after vagotomy before esophageal distension was tested.

Spinal cord transection (n = 6 animals). Acute denervation of the sympathetic nervous system was performed by first placing animals on a ventilator and then making two complete incisions through the exposed spinal cord at the level of the first cervical vertebra. These incisions were made laterally from opposite sides to ensure complete interruption of all descending fibers. An interval of at least 45 min was allowed after transection before the esophageal distension was tested. This interval was needed to ensure that the preparation had stabilized after spinal cord transection.

Intravenous N\textsuperscript{\textcircled{O}}-nitro-L-arginine methyl ester (n = 5 animals). In this study, two control responses were obtained for esophageal distension. Twenty-five minutes after the second control response, N\textsuperscript{\textcircled{O}}-nitro-L-arginine methyl ester (l-N\textsuperscript{\textcircled{O}}-NAME) was administered in a bolus intravenous dose of 10 mg/kg. At the 5- and 30-min time points following l-N\textsuperscript{\textcircled{O}}-NAME administration, reflex testing was performed.

Intravenous atropine methylbromide (n = 4 animals). This drug was administered as an intravenous bolus injection of 0.5 mg/kg in four of the five animals that had been evaluated for the effects of l-N\textsuperscript{\textcircled{O}}-NAME on esophageal distension-induced gastric relaxation. Ten minutes after atropine methylbromide was administered, esophageal distension was repeated.

TTX microinjection in mNTS (n = 6 animals). For these studies, esophageal distension was performed and the decrease in IGP noted. Distension was repeated using a 10-min interval. Once two stable control responses were obtained, TTX (10 pmol) was microinjected bilaterally into the mNTS. Five minutes later, the esophagus was distended and the decrease in IGP noted.

Once the general neural circuit involved in the esophageal distension-induced reflex was established, we proceeded to the studies of c-Fos expression and phenotyping of c-Fos expression (n = 7 experiments). Parallel studies were performed with intravenous nicotine (n = 5 experiments).

Next, microinjection studies of kynurenic acid (NTS, 6 experiments; DMV, 3 experiments), l-N\textsuperscript{\textcircled{O}}-NAME (mNTS, 5 experiments; DMV, 3 experiments); \alpha\textsubscript{2}-adrenergic receptor blocking agents (DMV, 7 experiments; mNTS, 6 experiments), and bicuculline (DMV, 3 experiments) were performed. Before performing these drug studies, we would locate the target site (either NTS or DMV) by microinjecting 500 pmol of L-glutamate. The NTS location would be reflected by noting a decrease in IGP with L-glutamate, whereas the DMV location would be reflected by noting the opposite response. After, at least two microinjections of 500 pmol of L-glutamate separated by at least a 5- to 10-min interval were tested to show reproducibility of the response.

Drugs

All of the following drugs were purchased from Sigma (St. Louis, MO): \alpha\textsubscript{1}-chloralose, atropine methylbromide, nicotine hydrogen tartrate, bicuculline methiodide, kynurenic acid, l-N\textsuperscript{\textcircled{O}}-NAME, TTX, urethane, yohimbine hydrochloride, and L-arginine hydrochloride. Dextemethasone was purchased from Elkins-Sinn (Cherry Hill, NJ). SKF 86466 was a gift from Dr. Paul Heible (Glaxo-SmithKline Beecham Pharmaceuticals, King of Prussia, PA). Sodium nitroprusside was purchased from Abbott Labs (North Chicago, IL). All drugs were dissolved in 0.9% saline except yohimbine, which was dissolved in double-distilled water and sonicated for 2–3 min. The pH of drug solutions in microinjection and intravenous studies was 7.0–7.2 and 7.0–7.4, respectively.

Rationale for Drug Doses Used

Information about drug doses and the rationale for selecting specific doses is provided in a previous report (12). This includes information on nicotine hydrogen tartrate, kynurenic acid, l-N\textsuperscript{\textcircled{O}}-NAME, L-arginine, yohimbine, SKF 86466, and bicuculline. The dose of TTX used for microinjection into the NTS was the same dose that was effective in an earlier study published by our laboratory (54). The dose of sodium nitroprusside used was the same as used in the study of Ishiguchi et al. (19). The dose of atropine methyl bromide used (0.5 mg/kg iv) was assumed to selectively block muscarinic receptors in the periphery.

Data Analysis

In all cases, statistical analysis was performed on both raw data and percentage of change. Paired sample t-test was performed when animals served as their own controls. Independent-sample t-test was performed on data from separate control and experimental test groups. Comparison between more than two means from different groups of rats was made by two-way ANOVA followed by post hoc analysis using the Newman-Keuls test. Differences were considered significant at P < 0.05. All values are expressed as means ± SE.

RESULTS

Effect of Bilateral Cervical Vagotomy, Spinal Cord Transection, Intravenous l-N\textsuperscript{\textcircled{O}}-NAME, and CNS Microinjections of TTX on Esophageal Distension-Induced Decreases in IGP

In six neurally intact rats, distension of the thoracic esophagus produced significant decreases in IGP, and a representative experimental tracing from one of these animals is shown as Fig. 1A. The magnitude of the decrease in IGP was linearly related to the volume of distension of the thoracic esophagus (Fig. 1B). A decrease in IGP was noted in some animals when the volume of distension was 0.6 ml and decreased further when the volume of distension was increased in roughly 0.1-ml increments up to 1.0 ml (Fig. 1B). Bilateral cervical vagotomy was performed in six animals, and IGP was monitored during distension of the thoracic esophagus. Data are presented in Fig. 1B and indicate that vagotomy completely prevented esophageal distension from decreasing IGP. Experimental tracings of one representative animal showing the blocking effect of bilateral cervical vagotomy on esophageal distension-induced decrease in IGP are shown in Fig. 1A. Spinal cord transection was performed in six animals, and IGP was again monitored during distension of the thoracic esophagus. Data are summa-
rized in Fig. 1 B and indicate that spinal cord transection had no
effect on the relationship between esophageal distension and
decreases in IGP.

In an additional five neurally intact rats, two control re-
sponses were obtained for esophageal distension-induced de-
creases in IGP. At 5 and 30 min after L-NAME administration,
reflex testing was performed. At these two time points, esopha-
geal distension-induced decreases in IGP were present and of
the same magnitude as the control responses. A representative
experimental tracing from one of the five animals studied is
shown in Fig. 2. As shown, L-NAME pretreatment had no
effect on the decrease in IGP evoked by esophageal distension.
This was true in five of five animals at both 5 and 30 min after
L-NAME administration. In four of the five animals, atropine
methylbromide was administered. Ten minutes later, esopha-
geal distension no longer evoked a decrease in IGP (Fig. 2D).
Atropine methylbromide always reduced the baseline IGP.
Hence, it could be argued that after this agent was given, IGP
might not drop any further. To test this, sodium nitroprusside
in a dose of 50 μg/kg was administered as an intravenous bolus
injection and was found to always elicit a robust decrease in
IGP (e.g., see Fig. 2E). The summarized data for these studies
in five neurally intact rats indicate that esophageal distension
decreased IGP by 0.25 ± 0.04 mmHg (P < 0.05). The
decreases in IGP elicited by esophageal distension 5 and 30
min after intravenous L-NAME administration were 0.45 ±
0.06 and 0.51 ± 0.05 mmHg (P < 0.05), respectively. With
intravenous atropine methylbromide administration, no signif-
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deferences in IGP after esophageal distension. Five animals were
also studied using vehicle for TTX, i.e., 0.9% physiological
saline. Vehicle microinjected bilaterally into the mNTS had no
effect on the esophageal vagovagal reflex (Fig. 3). Microinjec-
tion sites for these studies are depicted as part of Fig. 10.

Fig. 1. A: representative experiment showing the response of intragastric pressure (IGP) to 1 min of esophageal distension (indicated by horizontal bar) before (control) and after bilateral cervical vagotomy. Note the absence of esophageal distension-induced decrease in IGP after vagotomy. B: effects of esophageal distension volume on IGP of neurally intact animals (n = 6), vagotomized animals (n = 6), and spinal cord-transected animals (n = 6). Data plotted are means; vertical bars indicate SE.

Fig. 2. Representative experiment showing the effect of Nω-nitro-arginine methyl ester (L-NAME) and atropine methylbromide on the response of IGP to 15 s of esophageal distension (indicated by horizontal bar). Trace A is the control response. Traces B and C are IGP responses obtained 5 and 30 min after L-NAME (10 mg/kg iv), respectively. Note the lack of blockade of esophageal distension-induced decrease in IGP by L-NAME. Trace D is the IGP response obtained at 10 min after atropine methylbromide (0.5 mg/kg iv). Note esophageal distension no longer decreases IGP after blockade of muscarinic receptors. Also note that after atropine methylbromide, the baseline IGP has decreased. Trace E shows the effect of sodium nitroprusside (50 μg/kg, indicated by arrow) on IGP. The robust decrease in IGP observed with sodium nitroprusside indicates that IGP is still capable of decreasing, although baseline IGP has been lowered by atropine methylbromide.
Effect of Esophageal Distension on c-Fos Expression in Brain Stem Nuclei and Comparison With Intravenous Nicotine

In a previous study in our laboratory, c-Fos expression was used as a marker of neuronal activation to identify hindbrain sites where intravenous administered doses of nicotine that selectively reduced gastric tone acted (12). These nicotine doses produced significant increases in c-Fos expression in the NTS area compared with the effect of saline vehicle.

Seven animals underwent esophageal distension as described in METHODS, and five animals served as controls (i.e., a catheter was placed in the thoracic esophagus, but the balloon was not inflated). Data from a representative animal of each group are presented in Fig. 4. As shown, esophageal distension produced significant increases in c-Fos expression in the NTS (Fig. 4, distension) relative to the effect of no esophageal distension (Fig. 4, sham). c-Fos expression occurred throughout the rostrocaudal extent of the NTS and generally dorsal to the DMV. The c-Fos-labeled cells were too diffusely distributed throughout the NTS for us to conclude that any particular subdivision was selectively excited. c-Fos data from all animals tested are summarized in Fig. 5 and provide quantitative values for brain sections taken at 150-μm intervals. The profile of esophageal distension-induced activation of neurons indicates that significant excitation occurred from +1.05 mm rostral to CS to −0.6 mm caudal to CS, with the maximal number of NTS neurons activated at the CS.

Fig. 3. Microinjection of tetrodotoxin (TTX; 10 pmol) bilaterally into the medial subnucleus of the tractus solitarii (mNTS) attenuates the decrease in IGP elicited by esophageal distension. *P < 0.05 vs. control.

**Effect of Esophageal Distension on c-Fos Expression in Brain Stem Nuclei and Comparison With Intravenous Nicotine**

Fig. 4. Distribution of c-FOS-labeled cells in the nucleus tractus solitarii (NTS) and the dorsal motor nucleus of the vagus (DMV) after esophageal distension. A–F: representative photomicrographs showing the rostrocaudal presence of c-FOS (black nuclei) and retrogradely labeled Fluoro-Gold neurons (yellow; intraperitoneal injection) staining in a sham animal (A–C) and in an animal that had its esophagus distended (D–F). Note the relative absence of c-Fos-labeled cells in the photomicrographs at left and their concentration in the NTS at right. Calamus scriptorius (CS) is used as a reference point that designates the caudal tip of the area postrema (AP). Rostrocaudal distances of photomicrographs A, C, D, and F are in reference to CS. 4V, fourth ventricle; cc, central canal; AP, area postrema.
To identify the phenotype of neurons in the NTS expressing c-Fos in response to esophageal distension, we used the information provided by pharmacological studies of a previous nicotine study (12). Data from that study indicated that noradrenaline and nitric oxide were important signaling molecules in CNS pathways of a vagovagal reflex-induced inhibition of the gastric fundus. Hence, we posed the question of whether NTS neurons excited by esophageal distension similarly exhibited immunoreactivity to TH and to NOS, markers for catecholaminergic neurons and neurons capable of synthesizing nitric oxide, respectively. Studies of TH immunoreactivity indicated that a statistically significant number of neurons were excited, and this is shown in Fig. 6. The highest number of c-Fos(+/TH-IR) neurons was located 0.30 to 0.60 mm rostral to CS. As part of Fig. 6, photomicrographs of c-Fos and TH staining at two magnifications in a representative section +0.5 mm rostral to CS are included.

The total number of TH-IR neurons activated by esophageal distension relative to the total number of TH-IR neurons present was <10%, similar to that reported by Willing and Berthoud (57) for distension of the stomach. In our study, only 39 ± 3 TH neurons were excited on the basis of the presence of c-Fos, whereas the total number of TH neurons present in the rostrocaudal span of the NTS from −0.60 mm caudal to +1.05 mm rostral to CS was 582 ± 34, i.e., 6.7 ± 0.5%. However, examination of each 150-μm section of brain stem on either side of CS indicated that at one level rostral to CS, as many as 19.7 ± 2.3% of the TH neurons present were activated (Table 1). The rostrocaudal location of the TH-IR cells activated corresponds to the A2 or norepinephrine group of neurons as described anatomically by Paxinos et al. (31).

Another important finding was in regard to data obtained for the DMV. Esophageal distension, while exciting neurons in the NTS area based on c-Fos expression, had relatively little effect on c-Fos expression in DMV neurons that projected to the periphery (Table 2). Counts for c-Fos expression in retrogradely labeled FG(+) DMV neurons in rats exposed to esophageal distension and to placement of the uninflated balloon cannula (sham control animals) were either physiologi-

Fig. 5. Rostrocaudal distribution of c-FOS-labeled cells in the NTS after sham or esophageal distension. *P < 0.05. Significance is based on comparison between treatments in comparable sections using 2-way ANOVA.

Fig. 6. A: rostrocaudal distribution of cells double-labeled for c-Fos expression and tyrosine hydroxylase (TH) immunoreactivity (cFOS+TH) in the NTS after esophageal distension. B: photomicrograph of c-FOS and TH staining in a representative section +0.5 mm rostral to CS. C: higher magnification of stippled area in B showing single- (TH; double arrows) and double-labeled (c-FOS+ TH; arrows) cells in the NTS. Note that black nuclei are single c-Fos-labeled cells; 12n, hypoglossal nucleus. *P < 0.05. Significance is based on comparison between treatments in comparable sections using 2-way ANOVA.
cally insignificant (0.002% of the total DMV neurons projecting to the stomach) or zero, respectively (Table 2).

As mentioned above, data on the effects of intravenous doses of nicotine on c-Fos expression in brain stem nuclei were reported by our group previously (12). As indicated above, these studies were conducted in conscious rats, and nicotine was given intravenously in doses of 56.5, 113, and 226 nmol/kg. The increases in c-Fos expression that occurred were similar for all three doses of nicotine, and data were tabulated as the average number of neurons exhibiting c-Fos per transverse section (12). To compare results of intravenous nicotine to esophageal distension, we have combined data from all three doses of nicotine and expressed the results as histograms reflecting the number of neurons in 150-μm transverse sections ranging from +1.05 mm rostral to CS to −0.9 mm caudal to CS (Fig. 8A). Five animals composed each study group. As can be noted, a similar pattern of c-Fos activation occurred in the NTS with nicotine as it did with esophageal distension (compare data of Fig. 8A with data of Fig. 5).

In our earlier study, we did not assess the phenotype of NTS neurons exhibiting c-Fos from intravenous nicotine exposure. Those brains from nicotine-treated rats were processed for TH and NOS immunoreactivity and for c-Fos. The results obtained indicate that nicotine, like esophageal distension, activated TH-IR neurons. The rostral caudal NTS distribution of c-Fos(+)/TH-IR neurons was similar to the profiles observed with esophageal distension (compare data of Fig. 8B with data of Fig. 6A). The total number of TH-IR neurons activated over the rostrocaudal span shown in Fig. 8B was 35 ± 14. The total number of TH-IR neurons in this rostrocaudal span was 728 ± 88. Thus the percentage of total immunoreactive neurons activated by nicotine was 4.9 ± 1.5%. However, examination of each 150-μm section of brain stem on either side of CS indicated that at one level rostral to CS as many as 19.7 ± 4.2% of the TH neurons present were activated (Table 1).

We did not observe a significant activation of NOS-IR neurons after nicotine administration (data not shown). This analysis was performed in brain stems of four saline control animals and five nicotine-treated animals. Finally, in regard to the effects of intravenously administered nicotine in doses of 56.5, 113, and 226 nmol/kg on DMV neurons, investigators in our group already reported in a previous study that these doses did not activate FG retrogradely labeled DMV neurons projecting to the stomach (12).

**Table 1. Effect of either esophageal distension or intravenous nicotine on percentage of TH neurons activated at different rostrocaudal levels of the NTS**

<table>
<thead>
<tr>
<th>Location of 150-μm Section Relative to CS, mm</th>
<th>Esophageal Distension (n = 7)</th>
<th>Intravenous Nicotine IV (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of neurons exhibiting both TH immunoreactivity and c-Fos</td>
<td>Total no. of TH-IR neurons present</td>
<td>TH neurons activated, %</td>
</tr>
<tr>
<td>No. of neurons exhibiting both TH immunoreactivity and c-Fos</td>
<td>Total no. of TH-IR neurons present</td>
<td>TH neurons activated, %</td>
</tr>
<tr>
<td>+1.05</td>
<td>2 ± 0</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>+0.90</td>
<td>3 ± 1</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>+0.75</td>
<td>2 ± 1</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>+0.60</td>
<td>7 ± 1</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>+0.45</td>
<td>6 ± 2</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>+0.30</td>
<td>7 ± 1</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>+0.15</td>
<td>5 ± 0</td>
<td>68 ± 5</td>
</tr>
<tr>
<td>0 (CS)</td>
<td>3 ± 1</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>−0.15</td>
<td>2 ± 1</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>−0.30</td>
<td>2 ± 1</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>−0.45</td>
<td>0</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>−0.6</td>
<td>0</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>−0.75</td>
<td>0</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>−0.9</td>
<td>0</td>
<td>27 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of animals. TH, tyrosine hydroxylase; NTS, nucleus tractus solitarii; CS, calamus scriptorius; IR, immunoreactive.

**Effect of Bilateral Microinjection of Kynurenic Acid and L-NAME Into mNTS on Esophageal Distension-Induced Decrease in IGP**

The protocol used for the TTX experiments was employed for studies of kynurenic acid. Kynurenic acid (100 pmol) was microinjected bilaterally into the mNTS in six experiments and was found to significantly counteract the decreases in IGP produced by esophageal distension (Fig. 9A). Bilateral microinjection of kynurenic acid into the DMV of three animals exerted no significant antagonism of the reflex-induced response (Fig. 9A).

Similar experiments were carried out with L-NAME, and the data are summarized in Fig. 9B. L-NAME (45 nmol) microinjected bilaterally into the mNTS in five experiments and was significantly counteracted the decrease in IGP evoked by esophageal distension. Once L-NAME-induced antagonism was present in three of the five experiments, L-arginine (225 nmol) was microinjected bilaterally into the same site as L-NAME. Within a few minutes of L-arginine microinjection, there was reversal of L-NAME-induced antagonism of the vagovagal reflex (Fig. 9B). In the two experiments in which L-arginine was not microinjected, the L-NAME-induced antagonism persisted for at least 10 min beyond the time at which L-arginine had been administered. Finally, in three parallel experiments with L-NAME performed at the DMV, bilateral microinjection of 45 nmol had no effect on the reflex-induced response (Fig. 9B). The microinjection sites for kynurenic acid, L-NAME, L-arginine, and “saline control” into the mNTS are depicted in Fig. 10.
Effect of Bilateral Microinjection of α2-Adrenergic Receptor Blocking Agents and Bicuculline Into DMV on Esophageal Distension-Induced Decrease in IGP

In the first series of experiments, two drugs known to block α2-adrenergic receptors, namely, yohimbine and SKF 86466, were tested at the DMV to determine whether they would counteract the decrease in IGP produced by distension of the thoracic esophagus. In the second series of experiments, a drug known to block the GABA_A receptor, namely, bicuculline, was tested at this site. As shown in Fig. 9C, bilateral microinjection of either yohimbine (500 pmol; n = 4) or SKF 86466 (100 pmol; n = 3) into the DMV significantly counteracted esophageal distension-induced decreases in IGP. In contrast, the same dose of each of these agents bilaterally microinjected into the mNTS (n = 3 for each drug) had no effect (data not shown). A representative experiment showing the ability of yohimbine microinjected into the DMV to antagonize the reflex-induced response is shown in Fig. 10. The microinjection sites for yohimbine and SKF 86466 into the DMV and mNTS are depicted in Fig. 10. Data obtained with bicuculline (50 pmol) microinjected into the DMV in three experiments

**Table 2. Effects of esophageal distension on c-Fos activation in DMV neurons and in DMV neurons retrogradely labeled with Fluoro-Gold from the stomach**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>FG-Labeled DMV Neurons</th>
<th>c-Fos-Labeled Neurons in DMV Area</th>
<th>Double-Labeled (FG+c-Fos) DMV Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.280±186</td>
<td>11±7</td>
<td>0</td>
</tr>
<tr>
<td>Esophageal distension</td>
<td>1.677±47</td>
<td>28±6</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of animals. FG, Fluoro-Gold; DMV, dorsal motor nucleus of the vagus.

**Fig. 7.** A: rostrocaudal distribution of cells double-labeled for c-Fos expression and nitric oxide synthase (NOS) immunoreactivity (cFOS+NOS) in the NTS after esophageal distension. B: photomicrograph of c-FOS and NOS staining in a representative section +0.5 mm rostral to CS. C: higher magnification of stippled area in B showing single- (NOS; double arrows) and double-labeled (cFOS+NOS; arrow) cells in the NTS. Note that black nuclei are single c-Fos-labeled cells. *P < 0.05. Significance is based on comparison between treatments in comparable sections using 2-way ANOVA.

**Fig. 8.** Rostrocaudal distribution of c-FOS-labeled (A) or double-labeled cFOS+TH (B) cells in the NTS after intravenous infusion of either saline or nicotine. *P < 0.05. Significance is based on comparison between treatments in comparable sections using 2-way ANOVA.
did not counteract esophageal distension-induced decreases in IGP (data not shown).

**DISCUSSION**

Our data support the view that a noradrenergic pathway originating in the NTS and projecting to the DMV is part of the hindbrain circuitry for transmitting sensory information from the lower esophagus to the DMV. Evidence for this consists of multiple findings. First, esophageal distension excites TH-containing cells located in the NTS from CS to +0.90 mm rostral to CS. TH is a marker of neurons containing dopamine, norepinephrine, or epinephrine. Because the A2 norepinephrine group of neurons corresponds anatomically to the rostro-caudal region ranging from +0.90 mm to CS (31), we concluded that the c-Fos(+)/TH-IR NTS neurons activated in animals subjected to esophageal distension were most likely noradrenergic.

Additional evidence that esophageal distension activates noradrenergic neurons in the NTS that project to the DMV is that microinjection of α2-adrenoreceptor antagonists, namely, yohimbine and SKF 86466, into the DMV counteracted esophageal distension-induced decreases in IGP. Fukuda et al. (14), using a rat brain slice preparation, reported that norepinephrine activated α2-adrenoreceptors located on DMV neurons, which then resulted in inhibition of these neurons. Later confirmation of this result was made by Martinez-Peña et al. (24). This finding, together with our evidence, suggests that esophageal distension activates NTS noradrenergic neurons, causing release of norepinephrine, which then activates α2-adrenoreceptors and inhibits the cholinergic excitatory pathway to the stomach.

Consistent with our findings are data of other investigators providing anatomical and electrophysiological evidence that noradrenergic neurons connect the NTS to the DMV (5, 14, 33, 43). In fact, the DMV has been described as receiving more noradrenergic terminals than any other medullary nucleus (34). We assume that the A2 norepinephrine neurons are the important source of the terminals in our study, but there also is evidence that A6 norepinephrine neurons are another source of these terminals (48). We have singled out the A6 noradrenergic cell group from the noradrenergic cell groups, i.e., A1 and A5, because of the report that excitation of A6 neurons produces vagus nerve-mediated decreases in gastric motility (30). Rogers et al. (39) were the first to demonstrate that noradrenergic neurons in the rat NTS were activated by esophageal distension, and they provided data indicating that these neurons participated in the esophageal-gastric relaxation reflex. Immunohistochemical techniques were used and showed that esophageal distension excited 53% of TH-IR neurons in the central division of the NTS. Excitation was reflected by colocalization of the TH immunoreactivity with c-Fos. This activation of a high percentage of the total number of noradrenergic neurons in the NTS, combined with data showing that pharmacological blockade of α1- and α2-receptors in the dorsomedial medulla counteracted the esophageal-gastric relaxation, provided strong evidence that noradrenergic neurons in the nucleus tractus solitarii are a necessary component of this vagovagal reflex. In our study, we observed far fewer TH-IR neurons that were activated by esophageal distension. The maximal percent of the total noradrenergic neurons excited at a specific distance from the CS was 19.7 ± 2.3%. The reason for this finding probably relates to the differences in the intensity of the esophageal distension stimulus utilized. Rogers et al. (39) used repetitive distension for 90 min, whereas we used a protocol consisting mainly of three 1-min periods of distension. Because of the lower number of TH-IR neurons excited by our method of esophageal distension, we sought further confirmatory evidence for the participation of NTS noradrenergic neurons in the operation of a vagovagal reflex. Our earlier studies indicated that intravenous nicotine activated this reflex, resulting in gastric relaxation (12), and associated with gastric relaxation was an increase in c-Fos in the mNTS neurons. In addition, α2-receptor antagonists microinjected into the DMV blocked nicotine-induced gastric relaxation. This raised the question as to the phenotype of the NTS neurons that exhibited c-Fos after...
nicotine administration, and particularly whether a significant portion of those neurons were also TH immunoreactive. We therefore felt it was important for the interpretation of these studies to compare the percentage of TH-IR neurons activated by nicotine to our esophageal distension data. We found that nicotine activated the same general percentage of TH-IR neurons (19.7 ± 4.2%) as were activated by esophageal distension and, furthermore, that these activated neurons were localized to a similar rostrocaudal location in the NTS. Together, these data therefore suggest that activation of as little as one-fifth of the noradrenergic neurons in a specific rostrocaudal location of the mNTS is associated with vagovagal-mediated reflex-induced inhibition of the stomach.

Rogers et al. (39) reported that blockade of α2-adrenoceptors in the hindbrain area with yohimbine (which was applied to the floor of the 4th ventricle) reduced esophageal distension-induced fundic relaxation to 56% of control. Blockade of α1-adrenoceptors in the hindbrain with prazosin (also applied to the floor of the 4th ventricle) reduced esophageal distension-induced fundic relaxation to 55% of the control, and a combination of the two blockers reduced the distension-induced response to 28% of control. We found that α2-adrenoceptor blockade elicited a response in our study equivalent to the combination of combined α2-receptor and α1-receptor blockade in the study by Rogers et al. (39). On the basis of our results with α2-receptor blockade produced at the DMV, we conclude that activation of an NTS noradrenergic pathway projecting to the DMV results in synaptic release of norepinephrine that, in turn, activates predominantly α2-adrenoceptors on DMV neurons. In agreement are the findings of Rosin et al. (40), who described α2c-adrenergic receptor-like immunoreactivity located on the perikarya of DMV neurons of the rat. These α2-adrenoceptors (α2C subtype?) are located on cholinergic excitatory neurons, and activation results in inhibition of vagal outflow. Decreased vagal outflow then leads to relaxation of gastric smooth muscle tone and decreases in IGP. Consistent with our view are the findings of Fukuda et al. (14), demonstrating that excitation of the NTS noradrenergic pathway to the DMV only affects α2-adrenoceptors on DMV neurons and not α1-adrenoceptors on the same neurons (presumably, the α1-adrenoceptor is extrasynaptic). Also in support of sole inhibition of DMV neurons by the NTS noradrenergic projection is the finding that c-Fos expression was not activated in DMV neurons after esophageal distension, similar to previous findings after nicotine stimulation (12).

Our data obtained with bilateral microinjection of kynurenic acid into the NTS demonstrated antagonism of esophageal distension-induced decreases in IGP. These data indicate that L-glutamate acting on ionotropic receptors is involved in mediating the reflex-induced response. It is known from studies of McCann and Rogers (25) that glutamate is released from vagal afferent nerves, which could directly excite second-order NTS noradrenergic neurons.

Because some responses to glutamate are mediated by nitric oxide (15, 50), we evaluated whether this signaling molecule might act as a link between released glutamate and NTS noradrenergic neurons. First, we assessed whether esophageal distension would activate NTS neurons that express NOS. Our c-Fos expression studies combined with NOS immunoreactivity indicated that only a small number of neurons were double labeled with c-Fos and NOS immunoreactivity, which is unlikely to be of physiological significance. Second, we assessed whether microinjection of L-NAME, an inhibitor of NOS, into the mNTS area would counteract the esophageal distension-induced decrease in IGP. The magnitude of the antagonism was equal to the antagonism observed with kynurenic acid and TTX microinjected into the same site. Microinjection of the same dose of L-NAME into the nearby DMV had no effect on reflex-induced decrease in IGP. Microinjection of L-arginine into the mNTS at the peak of the block with L-NAME reversed the L-NAME-induced antagonism. The lack of effect of L-NAME in the DMV and the reversal of the blockade with L-arginine microinjections into the mNTS strongly suggests
that L-NAME was acting at the NTS and its blocking effect was due to inhibition of synthesis of nitric oxide.

Results obtained using nicotine to activate a vagovagal reflex were similar to those obtained with esophageal distension. In the present study, intravenous nicotine did not excite NTS neurons that were immunoreactive for NOS. On the other hand, microinjection studies reported previously by our group (12) indicated that L-NAME microinjected into the NTS blocked nicotine-induced effects on IGP and that L-arginine microinjected into the same site reversed the blockade.

To reconcile the findings of a lack of activation of NOS-containing NTS neurons during esophageal distension, as assessed by c-Fos expression, with the ability of L-NAME microinjected into the mNTS to counteract the reflex-induced decreases in IGP, we suggest that glutamate, instead of exciting the cell bodies of NOS neurons, may instead excite NOS-containing nerve terminals in the NTS neuropil. The site where vagal afferent nerves link the esophagus to the NTS is the subnucleus centralis, which is defined by the high density of NADPH-diaphorase-stained terminals and cell bodies within it (22, 31). It is known that presynaptic nerve terminals can serve as a source of nitric oxide and that stimulation of glutamate receptors on terminals can lead to nitric oxide production (28).

We speculate that glutamate released from vagal afferent terminals synapsing in the subnucleus centralis activates glutamate ionotropic receptors on NOS-containing terminals. This causes nitric oxide to be formed at the terminals and then released, where it then acts intercellularly to excite nearby noradrenergic projection neurons that synapse in the DMV (Fig. 11). Nitric oxide could also act as a retrograde messenger (27) to evoke additional release of glutamate from nearby vagal afferent terminals (Fig. 11).

The reason for the significant differences between our findings and those of Rogers et al. (39) need to be addressed. Two major differences were 1) in our study, blockade of α2-adrenoreceptor at the DMV largely abolished the vagovagal reflex, whereas only a partial blockade was observed in the other study; and 2) inhibition of nitric oxide synthesis in the NTS in our study also abolished the reflex, whereas inhibition of NOS in the hindbrain in the other study was ineffective in altering the reflex. These differences may be related to the different methods for applying drugs to the NTS and DMV neurons. In our study using antagonists to the α2-adrenoreceptor and an inhibitor of NOS, these agents were microinjected using a volume of 60 nl into the specific areas of interest (i.e., NTS and DMV). Rogers et al. (39) delivered their antagonist via the floor of the fourth ventricle. Drug applied (micro-dropped in 2-μl volume) to the surface of the fourth ventricle would have ready access to neurons of the area postrema (AP) and NTS, as well as the DMV. Because α2-adrenoreceptors are present in the AP (4) and both α2-adrenoreceptors and nitric oxide are present in the NTS (10, 11, 49), it is difficult to interpret the data obtained with microdropping the drug in a 2-μl volume onto the dorsal surface of the medulla. The reason given for microdropping the drug onto the brain surface was that microinjection of the antagonist in the DMV would only cover a small portion of the total area of the DMV. Because of their earlier anatomical findings showing that NTS neurons in the ventral commissural nucleus of the tractus solitarii (TS) (36) and the central subnucleus of the TS (35) project throughout the entire anterior-posterior extent of the DMV, it seems reasonable to be concerned that an uninterpretable “negative result” would occur with drug microinjected into locally into DMV tissue. We did not have this concern for several reasons. First, in a previous nicotine study, investigators in our group were successful in obtaining “positive results” with microinjections of antagonist drugs (12). Second, the second-order neuron we appear to be dealing with is noradrenergic, and no data exist that describe the length of DMV that is innervated by noradrenergic terminals. Third, retrograde tracing studies show that the density of DMV neurons that project specifically to the fundus in the rat is greatest at the site of our microinjection and drops off precipitously in rostral and caudal areas of the DMV (29).

The other major difference between our findings and those of Rogers et al. (39) is that our data led us to conclude that the NANC vagal pathway plays no role in mediating esophageal distension-induced gastric relaxation. The data that supports this position are 1) intravenously administered L-NAME had no effect on distension-induced decrease in IGP (Fig. 2); and 2) an absence of c-Fos expression in DMV neurons that project to peripheral structures. Rogers et al. did not evaluate L-NAME against esophageal distension-induced gastric relaxation. In addition, they did not address whether gastric projecting DMV neurons exhibit c-Fos after distension of the esophagus. Instead, their evidence for involvement of a NANC pathway is indirect, consisting of data obtained by microdropping prazosin onto the floor of the fourth ventricle. They suggest that α1 antagonism blocks the direct postsynaptic effects of norepinephrine to activate the NANC component of DMV projections to the stomach. This suggestion is at odds with the findings of Fukuda et al. (14), who reported that excitation of the NTS noradrenergic pathway to the DMV only affects...
α2-adrenoceptors on DMV neurons and not α1-adrenoceptors. Other differences between the two studies were that of anesthesia and species of rats. In the study by Rogers et al., experiments were performed on thiobutabarbital-anesthetized Long-Evans (Charles River) rats, whereas our studies were carried out on urethane-chloralose- and/or pentobarbital-anesthetized Sprague-Dawley (Taconic) rats.

It should be noted that our study has several potential limitations, but these limitations, in our opinion, do not impact adversely on our findings. One limitation is that our experimental studies were conducted under three different experimental conditions. The functional studies were carried out using a mixture of urethane and α-chloralose. The c-Fos response to esophageal distension studies was carried out using pentobarbital. The reason for this is that anesthesia had to be used because the stress involved with stretching the thoracic esophagus in conscious animals was assumed to be great (see METHODS). Pentobarbital was used because urethane induces c-Fos expression (47). The nicotine studies were carried out in conscious rats because the minor experimental intervention did not put the animals under duress. Even though the experimental interventions differed, the c-Fos response evoked by esophageal distension and intravenous nicotine were of similar magnitude and pattern in the NTS, indicative of roughly equivalent reflex-induced activation of this part of the hindbrain. Furthermore, Rogers et al. carried out functional studies in barbiturate-anesthetized animals, specifically, thiobutabarbital-anesthetized rats, and observed both c-Fos in the NTS and esophageal distension-induced gastric relaxation (39). In their earlier study (35), Rogers et al. used rats anesthetized with urethane in a dose that was described as producing “a deep plane of anesthesia,” and these animals exhibited gastric relaxation with esophageal distension. Thus deep anesthesia does not abolish this vagovagal reflex. Another potential limitation is the weight we have placed on the c-Fos data obtained in the DMV neurons that project to the periphery. We have argued that the lack of c-Fos expression in these neurons during esophageal distension and nicotine administration indicates that DMV neurons are not excited by these two interventions. However, as pointed out in an earlier review (17), “absence of c-Fos expression does not necessarily mean that stimulation did not occur.” It should be pointed out, however, that our c-Fos methodology clearly is able to detect excitation of DMV neurons that project to the stomach. That is, we recently published results showing excitation of DMV neurons based on c-Fos expression after insulin-induced hypoglycemia (42). Furthermore, we also reported that cholecystokinin given intravenously also activates DMV neurons as determined by c-Fos expression (42). Interestingly, DMV neurons expressing c-Fos after cholecystokinin did not project to the stomach.

A final potential limitation and/or concern is that although intravenous L-NAME had no effect on esophageal induced gastric relaxation, microinjection of L-NAME into the mNTS did counteract this reflex-evoked response. Presumably, when L-NAME is administered intravenously in a dose of 10 mg/kg, the concentration of drug within the mNTS never rises to that attained with local microinjection of the drug.

In summary, on the basis of our present findings, we speculate that the scheme depicted in Fig. 11 represents the pathways and CNS signaling molecules by which esophageal distension and systemically administered nicotine evoke their effects on gastric motility. Both stimuli release glutamate in the NTS, with events initiated at sites I (distension) and 2 (nicotine activating a α1β2-nicotinic acetylcholinergic receptor; Ref. 12). Glutamate, in turn, activates an ionotropic receptor on NOS-containing terminals in the NTS, and nitric oxide acts to excite second-order noradrenergic neurons projecting to the DMV. Norepinephrine is released and activates α2-adrenoceptor on cholinergic excitatory neurons, causing them to hyperpolarize. This reduces ACh release at the ganglion and at the postganglionic parasympathetic nerve terminal. The subsequent reduction in stimulation of muscarinic postsynaptic receptors on gastric smooth muscle decreases IGP. Evidence that the esophageal distension reflex is a vagovagal mediated reflex was obtained by demonstrating that vagotomy but not spinal cord transection abolishes the decrease in IGP. Evidence that esophageal distension-induced decreases in IGP involves only inhibition of the cholinergic excitatory pathway and that the NANC inhibitory pathway likely plays no role was obtained from three types of experiments. First, atropine methylbromide completely blocked the end-organ response. Second, L-NAME, given intravenously in a dose that others have shown blocks the NANC pathway (19, 45) had no effect on the end-organ response. Third, esophageal distension, while exciting neurons in the NTS area based on c-Fos expression, had no effect on c-Fos expression in DMV neurons that project to the periphery.

GRANTS

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REFERENCES


