μ-Opioid receptor stimulation in the medial subnucleus of the tractus solitarius inhibits gastric tone and motility by reducing local GABA activity

Melissa A. Herman,1 Alisa Alayan,2 Niaz Sahibzada,2 Barbara Bayer,3 Joseph Verbalis,4 Kenneth L. Dretchen,2 and Richard A. Gillis2

1Interdisciplinary Program in Neuroscience, 2Department of Pharmacology, 3Department of Neuroscience, and 4Department of Medicine, Georgetown University, Washington, DC

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Herman MA, Alayan A, Sahibzada N, Bayer B, Verbalis J, Dretchen KL, Gillis RA. μ-Opioid receptor stimulation in the medial subnucleus of the tractus solitarius inhibits gastric tone and motility by reducing local GABA activity. Am J Physiol Gastrointest Liver Physiol 299: G494–G506, 2010. First published May 20, 2010; doi:10.1152/ajpgi.00038.2010.—We examined the effects of altering μ-opioid receptor (MOR) activity in the medial subnucleus of the tractus solitarius (mNTS) on gastric function in vivo. mNTS evoke changes in intragastric pressure (IGP), fundus tone, and the receptive relaxation reflex (RRR). Microinjection of the MOR agonists [d-Ala2,MePhe4,Gly-ol]enkephalin (DAMGO; 1–10 fmol) into the mNTS produced dose-dependent decreases in IGP. Microinjection of the endogenous MOR agonists endomorphin-1 and endomorphin-2 (20 fmol) into the mNTS mimicked the effects of 10 fmol DAMGO. Microinjection of 1 and 100 pmol DAMGO into the mNTS produced a triphasic response consisting of an initial decrease, a transient increase, and a persistent decrease in IGP. The increase in IGP appeared to be due to diffusion into the dorsal motor nucleus of the vagus. The effects of 10 fmol DAMGO in the mNTS were blocked by vagotomy and by blockade of MORs in the mNTS, which, in turn, inhibits ongoing local GABA activity and by reducing local GABA activity.

Address for reprint requests and other correspondence: R. A: Gillis, Dept. of Pharmacology, Georgetown Univ. Medical Center, 3900 Reservoir Rd., NW, Washington, DC 20057 (e-mail: gillisr@georgetown.edu).

GASTROINTESTINAL (GI) side effects represent a major adverse response to the use of opioid analgesics (4). Early studies suggested that both central and peripheral sites were involved (6, 25, 28). However, the current consensus is that activation of peripheral μ-opioid receptors (MORs) in the gut is largely responsible for adverse GI effects (5, 16, 23, 24). The primary reason for the shift in viewpoint to a predominantly peripheral site of action is data from experimental and clinical studies with opioid receptor antagonists thought not to cross the blood-brain barrier. These antagonists, namely methylnaltrexone and alvimopan, counteract the GI side effects of opioid drugs without antagonizing their analgesic effects (34). However, there are central sites not protected by the blood-brain barrier that are known to exert strong control over gastric function, and these “peripheral” antagonists could be acting at these unprotected sites.

Thus evidence of a peripheral site of action of opioid drugs based on the use of antagonists that are thought not to cross the blood-brain barrier is misleading. For example, nausea and vomiting are an adverse GI side effect that involves activation of MORs located in the area postrema, a circumventricular brain area that is unprotected by a blood-brain barrier (36). The opioid receptor antagonists methylnaltrexone and alvimopan also counteract nausea and vomiting associated with opioid analgesic use (11, 43, 46, 51), suggesting potential central effects of these drugs.

One potential central site of action is the medial subnucleus of the tractus solitarius (mNTS), an autonomic center that integrates gastric input from the periphery with higher brain centers. The mNTS is a good candidate for a central site of action of MOR agonists because it lacks an effective blood-brain barrier (17, 32) and contains MORs (30) as well as neurons that contain the endogenous MOR agonists endomorphin-1 and endomorphin-2 (EM-1 and EM-2) (33, 37). Electrophysiological studies performed in rat brain slice preparations demonstrate that the MOR agonists alter the synaptic activity of nucleus tractus solitarius (NTS) neurons (15, 39). Indeed, activation of MORs on the somatodendritic region of NTS GABA neurons suppresses their activity (14), leading investigators to suggest that an excitatory effect on gastric motility would occur (14). However, in vivo studies on gastric motor function in various species have shown opioids to delay gastric emptying and to inhibit gastric contractions (25).

For the above reasons, we hypothesized that the mNTS is an important central site where MOR activation influences GI motor function. In the present study, our primary goal was to examine MOR agonists and antagonists applied locally to the mNTS rather than MOR agonists and antagonists administered peripherally to assess whether they could exert effects at the mNTS area in the rat hindbrain. If positive effects on gastric tone and motility occurred, our goal was then to seek out the mechanisms responsible for MOR effects at the mNTS. Subsequent studies would then examine the importance of endogenous opioid signaling at the mNTS in a physiological reflex involving control of the fundus tone.

MATERIALS AND METHODS

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

All experiments were performed on adult male Sprague-Dawley rats (250–350 g) (Taconic). Animals were fasted overnight (18–24 h) with water available ad libitum.

Two experimental approaches were used: 1) microinjection of MOR agonists and antagonists into the mNTS to test effects on intragastric pressure (IGP) and 2) administration of MOR agonists and antagonists to test effects on the receptive relaxation reflex (RRR). Different anesthetics and different experimental end points for gastric motility were used for each approach.

In the studies testing microinjected drugs on IGP, the anesthetized was urethane (1.5 g/kg ip), since it has been reported not to augment GABA_{A} receptor signaling in the NTS (1). Adequate depth of anesthesia was assessed by the response to toe pinch and by the corneal reflex. Supplemental urethane (0.3 g/kg ip) was administered as needed. Body temperature was maintained at ~37°C with an infrared heating lamp. All animals were intubated to maintain a patent airway and to administer artificial respiration, if necessary. The carotid artery was cannulated with polyethylene tubing (PE-50) connected to a full-bridge pressure transducer (sensitivity: 5 μV·V\(^{-1}\)·mm·Hg\(^{-1}\)) for continuous monitoring of arterial blood pressure. The pressure transducer was connected to a bridge amplifier and a 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 cervical vagus nerves were isolated and individually looped with silk suture for selective sectioning during the course of the experiment.

To monitor gastric tone and phasic contractions, a low-compliance balloon constructed from a latex condom (connected to PE-60 tubing) was inserted into the stomach by way of the fundus and positioned toward the corpus/antrum area. The balloon was inflated with 3–5 ml of warm distilled water to produce global distension of the stomach. Preliminary experiments indicated that the baseline IGP produced by the 3–5 ml volume resulted in maximal contractile responses to microinjection of L-glutamate into the dorsal motor nucleus of the vagus (DMV). The tubing attached to the balloon was connected to a full-bridge pressure transducer (sensitivity: 5 μV·V\(^{-1}\)·mm·Hg\(^{-1}\)) that in turn was connected to a bridge amplifier and data-acquisition system (PowerLab; AD Instruments).

In the studies testing microinjected and intravenous drugs on the RRR, the anesthetic used was isoflurane, as it was the anesthetic used in our previous study involving the RRR (21). Isoflurane was administered via a nose cone (5% induction, 2–3% maintenance) vaporized with 95% oxygen and 5% CO\(_2\). After induction of anesthesia, the nose cone was switched to an intubation tube, subsequent to tracheotomy. Adequate depth of anesthesia was assessed as described above. Carotid artery and jugular vein cannulation were performed as described above.

To measure changes in fundus tone, a miniature strain gauge transducer (2 × 4 mm; RBI Products), calibrated before every experiment, was sutured onto the fundus, oriented along the circular smooth muscle fibers. The strain gauge was coupled to a bridge amplifier and data-acquisition system (PowerLab; AD Instruments). To provide uniform preload conditions, a low-compliance balloon constructed from a latex condom was inserted into the fundus through the pylorus 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 (21, 41) against which the fundus could relax. This preload strain was maintained for the duration of the experiment.

Microinjection Procedure

All animals were placed in a small animal stereotaxic frame (Kopf Instruments, Tujunga, CA) in a prone position. Following a partial craniotomy, the dura was reflected, and the underlying cerebellum was retracted to expose the dorsal medulla. Our goal was to place the micropipette tip in the area of the mNTS where the majority of gastric vagal afferents terminate (2, 44). Other areas of the NTS where gastric vagal afferents terminate are the subnucleus gelatinosus (2, 44), the dorsomedial NTS (8), and the subnucleus centralis (2). Since these subnuclei could also be affected by drug microinjected into the nearby mNTS, we refer to our microinjection site as the “mNTS area.” Microinjections were performed in either the right or left mNTS area. No difference in gastric motility responses was observed between the right or left mNTS so the microinjection data are pooled. The details of the micropipette technique used for delivering drugs including the stereotaxic coordinates are described fully in our previous paper (21).

To avoid any possible DMV effects of microinjected drugs, we performed unilateral microinjection into either the right or left mNTS of animals with the ipsilateral vagus nerve sectioned but with the contralateral vagus nerve intact, as described in our previous studies (7, 20). Because the DMV has only ipsilateral projections to the stomach, any effect of drug diffusion to the nearby DMV will have no functional impact on gastric motility. A diagram of our experimental model depicting the neurocircuity of the vagovagal pathways appears in our previous publication (20). Histological verification of microinjection sites was carried out for each experiment as described in our previous paper (20).

Esophageal Distension Technique

The esophageal distension (ED) technique was the same as that described in our earlier study (21). As in our earlier study, at the end of each experiment, examination of the esophagus confirmed that the distension balloon in each case was located in the thoracic esophagus.

Experimental Protocol for Studies Measuring Intragastric Pressure

A stable baseline IGP (reflecting the level of gastric tone and motility under the conditions of urethane anesthesia and with 3–5 ml of water injected into the IGP balloon) and a stable arterial blood pressure recording were obtained for at least 10 min before any experimetal manipulations were initiated. The micropipette was inserted, and each animal was allowed to stabilize for a period of at least 2 min. Thereafter, in most animals (exceptions are indicated below), L-glutamate (500 pmol/30 nl) was used as a tool to identify pipette location. When a decrease in IGP (i.e., reduction in gastric tone) was observed with L-glutamate microinjection, we assumed that the micropipette tip was placed correctly in the mNTS area. Prior to the microinjection of test drugs (i.e., MOR agonists and antagonists) into the mNTS, at least two reproducible L-glutamate responses were elicited, with a minimum interval of 10 min between each L-glutamate injection. Microinjected drug responses were compared with vehicle (0.9% saline) microinjection performed under identical conditions as the experimental treatments. In some experiments the drug treatments produced a persistent decrease in the baseline IGP recording. To determine whether a lack of response to a MOR agonist was not due to a loss of sensitivity in the IGP recording (i.e., a “floor effect”), we intravenously administered sodium nitroprusside (50 μg/kg) at the end of the experiment and noted a characteristic decrease in IGP, indicating that the recording system was still able to detect a decrease in IGP.

Studies where L-glutamate microinjection was used to identify the mNTS area, as described in a previous study (7), included studies in which only one experimental drug was studied (i.e., MOR agonist, MOR antagonist, and kynurenic acid). Studies in which L-glutamate was not used included all studies assessing two experimental microinjected drugs. In these studies, L-glutamate was not used because of the constraints of using a double-barreled pipette. These included studies of [D-Ala\(_2\),MePhe\(_4\),Gly(ol)\(_5\)]enkephalin (DAMGO) and MOR antagonists and interaction studies between DAMGO and either gabazine or kynurenic acid. At the time these studies were performed we had obtained consistent data demonstrating DAMGO-induced decreases in IGP. Hence, we used DAMGO-induced decreases in IGP to initially identify the mNTS area.
Experimental Protocol for Studies Measuring Changes in Fundus Tone With ED-Induced Activation of the RRR

Fundus tone (strain gauge recording) and blood pressure were monitored and recorded in all the experiments performed. A minimum interval of 10 min was used between application of a 2-ml gastric preload and the first ED. At least two reproducible responses to ED were obtained before the effects of experimental manipulations (e.g., microinjection or intravenous antagonist pretreatments) on ED responses were assessed. A 30-min interval between distensions was found to be sufficient to provide a consistent reproducible response to ED. At the end of each experiment, intravenous sodium nitroprusside (50 μg/kg) was 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.

For studies testing the effect of drugs on the RRR, two reproducible responses to ED were obtained with a 30-min interval between distensions. In the study of locally applied MOR antagonist, naltrexone (NLTX) (25 pmol/30 nl) was bilaterally microinjected into the area of the mNTS 20–25 min after the second reproducible response to ED. ED was then performed 5–10 min following bilateral microinjection of NLTX. ED was then repeated every 30 min for the next 1–1.5 h to observe any recovery from the effects of NLTX. In the study of locally applied MOR agonist, DAMGO (10 fmol/30 nl) was bilaterally microinjected into the area of the mNTS 20–25 min after the second reproducible response to ED. ED was then performed 5–10 min following bilateral microinjection of DAMGO. ED was then repeated every 30 min for the next 1–1.5 h to observe any recovery that occurred. In the study of systemically administered MOR antagonist, NLTX (10 mg/kg iv) was administered 10–15 min after the second reproducible response to ED. ED was then performed 15–20 min after NLTX administration. ED was then repeated every 30 min for the next 1–1.5 h to observe any recovery that occurred.

Drugs and Chemicals

The drugs and their concentrations or doses used were as follows: urethane (1.5 g/kg), L-glutamate (16.7 mM), DAMGO (0.003 μM to 3 mM), EM-1 (0.6 μM), EM-2 (0.6 μM), NLTX (0.83 mM and 10 mg/kg), SR-95231 (gabazine, 0.67 mM), kynurenic acid (33.3 mM), d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP; 33.3 μM), and sodium nitroprusside (50 μg/kg). All drugs were purchased from Sigma-Aldrich (St. Louis, MO) and were dissolved in 0.9% saline (pH 7.0–7.4).

Rationale for microinjected drug doses. DAMGO was microinjected in doses of 1 fmol/30 nl to 100 pmol/30 nl. The dose of 10 fmol/30 nl was chosen as our “standard” dose on the basis of studies demonstrating the dose response of DAMGO on GTPγS binding as an index of MOR activation (54). According to this study, the EC50 for DAMGO on GTPγS activation was 200 nM, a concentration equivalent to a 6 fmol/30 nl microinjection dose. We chose 10 fmol/30 nl because it is slightly higher than the EC50 concentration, but still is a subsaturating dose. EM-1 and EM-2 were microinjected at a dose of 20 fmol/30 nl. This dose was chosen as it is approximately equivalent to the dose of 10 fmol/30 nl of DAMGO, based on studies demonstrating that DAMGO is approximately twice as potent as EM-1 and EM-2 (22). Microinjection of all MOR agonists could be repeated following a period of 1 h (shorter intervals were not tested).

The 25 pmol/30 nl dose of NLTX was based on preliminary studies with naloxone (50 pmol/30 nl) (48) and the relative efficacy of NLTX compared with naloxone (49). The 1 pmol/30 nl dose of CTOP was based on a receptor activation study demonstrating the selectivity and relative potency of CTOP at 37°C (19) and on our preliminary studies using 300 fmol/30 nl. In these preliminary studies we found that 300 fmol did not consistently block the gastric effects of 1 pmol DAMGO microinjected into the mNTS.

The 20 pmol/30 nl dose of gabazine and the 1 mmol/30 nl dose of kynurenic acid were chosen on the basis of full antagonist properties demonstrated in our previous studies (20, 50).

Rationale for intravenous drug doses. The 10 mg/kg dose of NLTX was based on preliminary studies examining the effects of 1, 2.5, and 5 mg/kg NLTX on the RRR. At these doses no significant reduction in RRR response was observed. Hence, we used 10 mg/kg to carry out this study. This dose has also been used previously (52).

The doses of atropine methylbromide (0.1 mg/kg) and Nω-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg) were based on full antagonist properties demonstrated in our previous studies (7, 21).

Data Analysis

Data were analyzed by use of Chart software (AD Instruments). The end points used for gastric motility were IGP, which reflects the degree of tonic contraction of gastric smooth muscle, and phasic contractions, which were measured as the number of contractions in 1-min intervals. The end point used for fundus tone was gram tension, which reflects the degree of fundus tone. To measure inhibitory effects (i.e., decreases in gastric motility), values for IGP and fundus tone were always taken as the lowest value of the experimental tracing, even when phasic contractions were present. Changes in IGP, phasic contractions, or fundus tone in response to drug administration or ED were determined as the maximum change from a 3-min baseline recording just prior to microinjection. For experiments in which manipulations were performed twice (drug was microinjected twice or ED was performed twice under identical experimental conditions), the two responses were averaged.

Data are presented as means ± SE. A paired t-test was performed when animals served as their own controls. When animals represented independent samples, the data were analyzed by an unpaired t-test between separate control and experimental groups. A one-sample t-test was performed to determine whether a group mean was significant from zero. In addition, a one-way analysis of variance was performed when more than one experimental intervention was used followed by the Newman-Keuls post hoc test. In all cases, P < 0.05 was the criterion used to denote statistical significance. For dose-response data, a linear regression was performed and correlation coefficients were determined from slope and intercept values.

RESULTS

Effects of DAMGO Microinjection Into the Area of the mNTS on Gastric Tone and Phasic Contractions

Dose-response related effects were obtained by using a dose range of 1 to 10 fmol of DAMGO. Microinjection of 1 fmol into the mNTS area produced a small but significant decrease in IGP (0.09 ± 0.01 mmHg, P < 0.05; n = 3) and no change in mean arterial pressure (MAP). Microinjection of 5 fmol produced significant decreases in both IGP (0.38 ± 0.08 mmHg, P < 0.05; n = 3) and MAP (3.1 ± 1.7 mmHg; P < 0.05). The most robust effects occurred with 10 fmol DAMGO microinjected into the mNTS (0.61 ± 0.04 mmHg, P < 0.05; n = 35). MAP decreased by 8.8 ± 0.9 mmHg, P < 0.05; n = 35. The decreases in IGP and MAP occurred immediately after microinjection and reached a nadir in ~1 to 1.5 min. The average duration of the decrease in IGP was 2.6 ± 0.3 min, and the effect could be repeated following a 1-h interval. In four rats, the vehicle for DAMGO (0.9% saline) microinjected into the mNTS area had no effect on either IGP or MAP.

Data obtained for 10 fmol DAMGO and vehicle microinjection are summarized in Fig. 1A. An IGP recording from a
Effects of DAMGO Microinjection Into the DMV on Gastric Tone and Phasic Contractions

To attempt to understand the triphasic response observed with high doses of DAMGO microinjected into the mNTS, we next determined whether any component of the triphasic response could be due to diffusion to nearby areas known to influence gastric function. The most logical site for diffusion of a drug locally applied to the mNTS is the DMV. DAMGO (10 fmol) was microinjected into the DMV of four rats with both vagus nerves intact. Microinjection of DAMGO resulted in a significant increase in IGP (1.33 ± 0.48 mmHg; P < 0.05) that occurred immediately and was followed by a sustained period of increased contractility that lasted for 5–10 min. Microinjection of DAMGO had no effect on MAP. The effect of 10 fmol on IGP could be

representative experiment is shown as Fig. 1B. As can be noted, not only was there a decrease in tone, but there was also an inhibition of phasic contractions. Inhibition of phasic contractions occurred in 30 of the 35 rats studied. In five rats, no phasic contractions were present in the baseline IGP recording. The decreases in IGP and MAP obtained with 1, 5, and 10 fmol of DAMGO are graphed in Fig. 1, C and D. The microinjection sites for the experiments summarized in Fig. 1A are illustrated in Fig. 2. As can be noted, the micropipette tip in each case was located in the mNTS area, specifically in the area that runs parallel to the area postrema.

With doses above 10 fmol, the dose-response relationship was lost. That is, when 30 fmol of DAMGO was microinjected in six animals, responses were variable. Averaging all the IGP responses indicated that no significant change had occurred (+0.12 ± 0.25, P > 0.05; n = 6). The effect of 30 fmol DAMGO on MAP was also variable and averaging all responses indicated no statistical change in MAP. When 300 fmol was tested, the responses observed were still variable, but a triphasic pattern on IGP was seen to emerge. In the four rats studied, the pattern of response to microinjection of 300 fmol DAMGO was a brief decrease in IGP, followed by a transient increase in IGP, and finally an inhibition of IGP. However, averaging data obtained for each phase of the response resulted in no significant change in IGP. The effects of 300 fmol DAMGO on MAP were also variable and, when averaged, were not statistically significant.

When the dose of DAMGO was increased further to 1 pmol and 100 pmol, the triphasic pattern was more evident (see Fig. 3). Microinjection of 1 pmol DAMGO into the mNTS area in four rats resulted in an initial decrease in IGP (0.64 ± 0.11 mmHg, P < 0.05) and inhibition of phasic contractions (phase I); an increase in IGP (0.38 ± 0.07 mmHg, P < 0.05) and an increase in phasic contractions (2.9 ± 0.75 contractions/min, P < 0.05) (phase II); and a decrease in IGP (1.1 ± 0.35 mmHg, P < 0.05) with inhibition of phasic contractions (phase III). The initial decrease in IGP presumably had not reached its lowest level because it was interrupted by the second phase increase in IGP. We suggest this because the value attained for the decrease was no greater than the value attained with 10 fmol of DAMGO. In four additional rats, microinjection of 100 pmol of DAMGO resulted in a decrease in IGP (0.47 ± 0.08 mmHg, P < 0.05) and inhibition of phasic contractions (phase I); an increase in IGP (1.6 ± 0.61 mmHg, P < 0.05) and an increase in phasic contractions (3.8 ± 0.77 contractions/min, P < 0.05) (phase II); and a decrease in IGP (1.2 ± 0.29 mmHg, P < 0.05) with complete inhibition of phasic contractions (phase III). There was no statistically significant effect of 1 pmol and 100 pmol DAMGO on MAP. Representative traces of the high-dose effects of DAMGO microinjection on IGP and phasic contractions are depicted in Fig. 3. Histological examination of the brain indicated that the micropipette in each experiment was located in the mNTS area.

Effects of DAMGO Microinjection Into the DMV on Gastric Tone and Phasic Contractions

To attempt to understand the triphasic response observed with high doses of DAMGO microinjected into the mNTS, we next determined whether any component of the triphasic response could be due to diffusion to nearby areas known to influence gastric function. The most logical site for diffusion of a drug locally applied to the mNTS is the DMV. DAMGO (10 fmol) was microinjected into the DMV of four rats with both vagus nerves intact. Microinjection of DAMGO resulted in a significant increase in IGP (1.33 ± 0.48 mmHg; P < 0.05) that occurred immediately and was followed by a sustained period of increased contractility that lasted for 5–10 min. Microinjection of DAMGO had no effect on MAP. The effect of 10 fmol on IGP could be
Sectioning the remaining (contralateral) cervical vagus nerve on DAMGO-induced decreases in IGP, phasic contractions, and MAP was performed in 4 of the 35 rats receiving the 10 fmol dose of DAMGO. We focused on the 10 fmol microinjection dose because it corresponds to the EC50 concentration for DAMGO on GTPyS activation (54) and because consistent inhibitory effects on gastric motility were obtained (see Fig. 1). In these experiments, two DAMGO-induced decreases in IGP were obtained with only the vagus nerve contralateral to the mNTS microinjection site intact. An interval of 1 h was used between the two responses. Both responses were similar and therefore averaged (Fig. 5A). After 1 h of recovery following the second DAMGO-induced decrease in IGP, the remaining (contralateral) cervical vagus nerve was sectioned and microinjection of DAMGO was repeated. Prior to sectioning the remaining vagus nerve, microinjection of DAMGO resulted in a significant decrease in IGP (0.75 ± 0.12, P < 0.05) and phasic contractions. After the remaining cervical vagus nerve was sectioned, microinjection of DAMGO into the same site had no significant effect on IGP (0.05 ± 0.03 mmHg, P > 0.05). (Note: phasic contractions were always abolished by vagotomy, hence the effect of vagotomy on phasic contractions could not be assessed.) Contralateral vagotomy had no effect on DAMGO-induced decreases in MAP. The IGP data are summarized in Fig. 5A and a representative experimental tracing is shown as Fig. 5B. It can be noted in Fig. 5B that vagotomy reduced the baseline IGP. It could be interpreted that the loss of the IGP response to DAMGO was due to this repeated following a 1-h interval. After the ipsilateral vagus nerve was sectioned, microinjection of DAMGO into the DMV produced no significant change in IGP (−0.53 ± 0.34 mmHg). Data obtained for DAMGO microinjection into the DMV before and after ipsilateral vagotomy are summarized in Fig. 4A. An IGP recording from a representative experiment is shown as Fig. 4B. Histological examination of the brain indicated that the micropipette in each experiment was located in the DMV (Fig. 2).

When the dose of DAMGO microinjected into the DMV was increased to 1 pmol, the effects on gastric tone and motility increased. Microinjection of 1 pmol resulted in a significant increase in IGP (3.94 ± 1.08 mmHg; P < 0.05; n = 5). The increase in gastric tone and motility persisted for 10–15 min. Histological examination of the brain indicated that the micropipette in each experiment was located in the DMV.

These results are consistent with the idea that the second phase of the triphasic response evoked by microinjection of high-dose DAMGO (1 and 100 pmol) is due to diffusion to the DMV.

Efferent Pathways Mediating the Effects of DAMGO Microinjection Into the Area of the mNTS on Gastric Tone and Phasic Contractions

Fig. 2. Camera lucida drawings of unilateral microinjection sites for all experiments presented in Figs. 1A and 4A. Numbers on the left side of each coronal section indicate the distance of each section from calamus scriptorius. Microinjections were performed in both left and right hemispheres. For the purpose of clarity, DAMGO microinjections into the mNTS are shown only in the right hemisphere. DAMGO microinjections into the dorsal motor nucleus of the vagus (DMV) and vehicle microinjections into the mNTS are shown only in the left hemisphere. XII, hypoglossal nucleus; AP, area postrema; TS, tractus solitarius; VEH, vehicle.

Fig. 3. Representative experimental tracings depicting changes in IGP following unilateral microinjection of 1 pmol DAMGO (A) and 100 pmol DAMGO (B) into the mNTS. Roman numerals refer to specific phases of the triphasic effect on IGP.

AJP-Gastrointest Liver Physiol • VOL 299 • AUGUST 2010 • www.ajpgi.org
drop in baseline IGP. However, this was not the case since averaging all of the changes in IGP produced by vagotomy indicated that the change in baseline IGP was not statistically significant. Histological examination of the brain indicated that in each case the micropipette tip was located in the mNTS area (Fig. 2).

We next performed studies to determine whether the efferent pathway involved in the DAMGO-induced decrease in IGP was comprised of cholinergic excitatory, nonadrenergic-noncholinergic inhibitory, or both types of nerves. In these experiments, two DAMGO-induced decreases in IGP and phasic contractions were obtained with an interval of 1 h between the two responses. After the second reproducible response was obtained, either atropine methylbromide (0.1 mg/kg) or L-NAME (10 mg/kg) was intravenously administered. Ten minutes after the antagonist administration, DAMGO was microinjected into the mNTS area. In 4 of the 35 rats whose data are included in Fig. 1A, atropine methyl bromide was administered. Atropine per se produced a decrease in baseline IGP, similar to results obtained in our previous study (20). However, intravenous administration of sodium nitroprusside in the presence of atropine produced a decrease in baseline IGP, suggesting that the stomach still had the capacity to relax. Prior to atropine, microinjection of DAMGO significantly decreased IGP (0.82 ± 0.26 mmHg, \( P < 0.05 \)) and eliminated phasic contractions. After atropine administration DAMGO had no significant effect on IGP (0.03 ± 0.05 mmHg, \( P > 0.05 \)) or phasic contractions. In four additional rats whose data are summarized in Fig. 1A, L-NAME was administered. L-NAME per se had no effect on baseline IGP, consistent with data from our earlier study (20). Prior to L-NAME, microinjection of DAMGO significantly decreased IGP (0.43 ± 0.04 mmHg, \( P < 0.05 \)) and eliminated phasic contractions. After L-NAME administration DAMGO significantly decreased IGP (0.39 ± 0.05 mmHg, \( P < 0.05 \)) and phasic contractions. There was no significant difference between the IGP effect of microinjection of DAMGO performed before L-NAME and microinjection performed after L-NAME (\( P > 0.05 \)). Histological examination of the brain indicated that in each case the micropipette tip was located in the mNTS area.

These results indicate that DAMGO-induced inhibition of gastric motility is mediated by the vagal nerves, specifically through withdrawal of the cholinergic-cholinergic excitatory efferent pathway.

Effects of Endomorphin-1 and Endomorphin-2

Microinjection Into the Area of the mNTS on Gastric Tone and Phasic Contractions

Studies were performed using two additional MOR agonists, namely the endogenous agonists EM-1 and EM-2. Both EM-1 and EM-2 were microinjected into the mNTS at a dose of 20 fmol. Microinjection of EM-1 in five rats resulted in a significant decrease in IGP (0.50 ± 0.08 mmHg; \( P < 0.05 \)) and abolished phasic contractions. A small but significant decrease in MAP (4.4 ± 0.95 mmHg; \( P < 0.05 \)) was also noted. In two additional rats, microinjection of EM-1 at a higher dose (1 pmol), like DAMGO, produced a triphasic effect characterized by a decrease in IGP, followed by a transient increase in tone and phasic motility and ultimately resulted in a prolonged decrease in IGP and inhibition of phasic motility.

Microinjection of EM-2 in four rats also produced a significant decrease in IGP (0.50 ± 0.07 mmHg; \( P < 0.05 \)) and abolished phasic contractions. A small but significant decrease in MAP (4.8 ± 0.52 mmHg; \( P < 0.05 \)) was also noted. Histological examination of the brain confirmed that the micropipette tip in each case was located in the mNTS area.

Fig. 4. A: histograms of averaged IGP responses to unilateral microinjection of DAMGO (10 fmol/30 nl) into the DMV with both vagus nerves intact compared with microinjection of DAMGO after sectioning of the ipsilateral vagus nerve. *\( P < 0.05 \) paired t-test; \( n = 4 \). B: representative experimental tracing depicting changes in IGP following microinjection of DAMGO (10 fmol/30 nl) into the DMV with both vagus nerves intact (left trace) and after the sectioning of the ipsilateral vagus nerve (ipsi vx; right trace).

Fig. 5. A: histograms of averaged IGP responses to unilateral microinjection of DAMGO (10 fmol/30 nl) into the mNTS with the contralateral vagus nerve intact compared with microinjection of DAMGO after the remaining (contralateral) vagus nerve was sectioned (vx). *\( P < 0.05 \) paired t-test; \( n = 4 \). B: representative experimental tracing depicting changes in IGP following microinjection of DAMGO (10 fmol/30 nl) into the mNTS with the contralateral vagus nerve intact (left and middle traces) and after the remaining (contralateral) vagus nerve was sectioned (right trace).
**Effects of MOR Blockade in the Area of the mNTS on Gastric Tone and Phasic Contractions**

Two MOR antagonists were tested, namely NLTX and CTOP [a MOR selective antagonist (19)], to determine whether blockade of MORs would have any effect on either gastric tone or phasic contractions. Microinjection of NLTX (25 pmol) into the mNTS area in four rats produced no significant change in either IGP (−0.05 ± 0.02, P > 0.05), phasic contractions, or MAP (−2.4 ± 2.3, P > 0.05). In addition, microinjection of CTOP (1 pmol) into the mNTS area of four rats produced no significant change in either IGP (−0.03 ± 0.02, P > 0.05), phasic contractions, or MAP (−1.15 ± 1.3, P > 0.05). Histological examination of the brain indicated that the micropipette tip in each case was located in the mNTS area.

**Effect of MOR Blockade in the Area of the mNTS on DAMGO-Induced Decreases in Gastric Tone and Inhibition of Phasic Contractions**

Studies were performed to assess the effect of MOR blockade on the decreases in IGP and inhibition of phasic contractions produced by microinjection of the 10 fmol dose of DAMGO into the mNTS. In these experiments, two inhibitory responses to microinjection of DAMGO were first obtained with a 1-h interval between microinjections. Kynurenic acid (1 nmol) was microinjected into the same site 50 min after the second inhibitory response to DAMGO was obtained. Ten minutes after NLTX injection, DAMGO was microinjected. Prior to NLTX microinjection, DAMGO produced a significant decrease in IGP (0.70 ± 0.14 mmHg; P < 0.05), inhibition of phasic contractions, and a significant decrease in MAP (6.9 ± 0.55; P < 0.05). After NLTX microinjection, DAMGO had no significant effect on IGP (−0.03 ± 0.03 mmHg; P > 0.05), phasic contractions, or MAP (−2.1 ± 0.79; P > 0.05). Histological examination of the brain confirmed that the micropipette tip in each case was located in the mNTS area.

Studies were also performed to assess the effect of MOR blockade on the change in IGP produced by microinjection of a higher dose of DAMGO (1 pmol). Our goal was to determine whether all three phases of the changes in IGP produced by microinjection of 1 pmol DAMGO were mediated by activation of MORs. The MOR specific antagonist CTOP (1 pmol/30 nl) was first microinjected into the mNTS area of four rats. Five minutes after CTOP microinjection, DAMGO was microinjected into the same site. CTOP had no per se effect on IGP (−0.03 ± 0.02 mmHg; P > 0.05) or phasic contractions. In the presence of CTOP, DAMGO had no significant effect on either IGP (−0.05 ± 0.04 mmHg; P > 0.05) or phasic contractions. Histological examination of the brain indicated that the micropipette was located in the mNTS area.

**Effect of GABAA Receptor Blockade in the Area of the mNTS**

Two inhibitory responses to microinjection of DAMGO (10 fmol) into the mNTS area were first obtained with a 1-h interval between microinjections. Gabazine (20 pmol) was microinjected into the same site and 10 min later DAMGO was microinjected again. These experiments were performed in 5 of the 35 rats whose data are included in Fig. 1A. Prior to gabazine microinjection, microinjection of DAMGO produced a significant decrease in IGP (0.46 ± 0.02 mmHg, P < 0.05), inhibition of phasic contractions, and decrease in MAP (5.4 ± 1.1 mmHg, P < 0.05). In the presence of gabazine, microinjection of DAMGO had no significant effect on IGP (−0.04 ± 0.02 mmHg, P > 0.05), phasic contractions, or MAP (−1.1 ± 0.6 mmHg, P > 0.05). The IGP data are summarized in Fig. 6A. Representative experimental traces depicting the changes in IGP with microinjection of DAMGO before and after gabazine microinjection are shown in Fig. 6B. Histological examination of the brain confirmed that the micropipette tip in each case was located in the mNTS area (Fig. 2).

Microinjection of gabazine produced a decrease in IGP (1.2 ± 0.2 mmHg, P < 0.05), phasic contractions, and a decrease in MAP (10.0 ± 3.2 mmHg, P < 0.05), which were qualitatively similar to data from our previous study (20). The decrease in baseline IGP produced by gabazine could confound the interpretation of the observed block of the DAMGO-induced decrease in IGP. To determine whether any of the antagonistic effects of gabazine were due to the decrease in baseline IGP by gabazine per se, sodium nitroprusside (50 μg/kg iv) was administered at the end of each experiment. Sodium nitroprusside always produced a robust decrease in IGP (see Fig. 6B, *inset*), indicating that the stomach still had the capacity to relax after gabazine microinjection.

**Effect of Ionotropic Glutamate Receptor Blockade in the Area of the mNTS**

Two inhibitory responses to microinjection of DAMGO (10 fmol) into the mNTS area were first obtained with a 1-h interval between microinjections. Kynurenic acid (1 nmol) was microinjected into the same site followed by DAMGO microinjection 10 min later. These studies were performed in 4 of the 35 animals whose data are included in Fig. 1A. Prior to
microinjection of kynurenic acid, DAMGO produced a significant decrease in IGP (0.47 ± 0.04 mmHg; \( P < 0.05 \)), inhibition of phasic contractions, and a decrease in MAP (8.6 ± 3.4 mmHg; \( P < 0.05 \)). In the presence of kynurenic acid, microinjection of DAMGO had no significant effect on IGP (\(+0.10 ± 0.08 \text{ mmHg}; P > 0.05\)) phasic contractions, or MAP (\(-1.8 ± 0.8 \text{ mmHg}; P > 0.05\)). The IGP data are summarized in Fig. 7A. Representative experimental traces depicting the changes in IGP and phasic contractions with microinjection of DAMGO before and after kynurenic acid microinjection are shown in Fig. 7B. Histological examination of the brain confirmed that the micropipette tip in each case was located in the mNTS area.

When kynurenic acid was microinjected into the area of the mNTS after two inhibitory responses to microinjected DAMGO, it produced robust effects on its own. In these experiments, kynurenic acid was microinjected 50 min after the second dose of DAMGO and produced an increase in IGP (1.8 ± 0.31 mmHg; \( P < 0.05 \)) and MAP (16.0 ± 2.3 mmHg; \( P < 0.05 \)). It also initiated phasic contractions (Fig. 7B), with a frequency of 3.0 ± 0.2 contractions/min and an amplitude of 3.6 ± 0.7 mmHg above baseline conditions. These responses did not occur immediately but had an average latency of 2.9 ± 1 min, a peak effect at 9.6 ± 0.9 min, and an average duration of 22.3 ± 2.1 min.

In a separate series of studies, kynurenic acid (1 nmol) was microinjected into the area of the mNTS at a time when DAMGO-induced suppression of GABA signaling was near maximal, i.e., 5 min after DAMGO microinjection. Microinjection of kynurenic acid in three rats produced a significant increase in IGP (1.6 ± 0.5 mmHg; \( P < 0.05 \)), frequency of contractions (3.0 ± 0.4 contractions/min; \( P < 0.05 \)), and amplitude of contractions (10.6 ± 3.7 mmHg; \( P < 0.05 \)). The increases in tone and motility occurred immediately after microinjection, with peak effects occurring at 17.7 ± 2.6 min after microinjection. The average duration of the increases in tone and motility with microinjection of kynurenic acid in the presence of DAMGO was 33.2 ± 2.9 min. In addition, microinjection of kynurenic acid produced a significant increase in MAP (5.0 ± 0.5 mmHg; \( P < 0.05 \)).

Experiments were also conducted to ascertain whether or not kynurenic acid microinjected into the area of the mNTS of naive rats would have an effect on IGP. Microinjection of kynurenic acid (1 nmol) was performed in five naive animals and found to have no significant effect on IGP (\(+0.12 ± 0.08 \text{ mmHg}; P > 0.05\)) but did increase MAP (7.1 ± 2.4 mmHg; \( P < 0.05 \)). These data are summarized in Fig. 7C. Representative experimental traces depicting the change in IGP with microinjection of kynurenic acid in naive animals (left trace) and 5 min after DAMGO microinjection (right trace) are shown in Fig. 7D. Histological examination of the brain confirmed that the micropipette tip in each case was located in the mNTS area.

These results indicate the following: 1) Kynurenic acid pretreatment prevents DAMGO-induced decrease in gastric motility. This finding is similar to that from our earlier study in which kynurenic acid pretreatment prevented decreases in IGP produced by microinjection of the GABA\(_A\) receptor antagonist bicuculline methiodide (20). 2) Kynurenic acid microinjected into the mNTS area after DAMGO produces an increase in gastric motility, similar to our previous study demonstrating that kynurenic acid increased gastric motility after microinjection of the GABA\(_A\) receptor antagonist gabazine (20). The increase in gastric motility was noted 5 and 50 min after DAMGO microinjection, but the effect was more robust at the 5-min time point, suggesting that the suppression of local GABA was present at both 5 and 50 min but had begun to wear off after 50 min. 3) Kynurenic acid microinjected into the mNTS of rats that had not received DAMGO had no effect on gastric motility. Presumably the lack of effect was due to ongoing GABA signaling canceling out all incoming gluta-

teric sensory input from the GI tract.

**Effect of Bilateral Microinjection of NLTX Into the Area of the mNTS on Reflex-Induced Decreases in Fundus Tone**

Distension of the esophagus was performed in seven rats with a balloon inserted into the thoracic esophagus and produced an inhibition of fundus tone (\(-0.23 ± 0.02 \text{ g}; P < 0.05\)).
ED was performed twice with a 30-min interval between distensions. Results from distensions were similar and therefore averaged. Twenty minutes following the second ED, NLTX (25 pmol) was bilaterally microinjected into the mNTS area. Microinjection of NLTX had no significant effect on fundus tone or MAP. ED performed in the presence of microinjected NLTX produced no significant inhibition of fundus tone (−0.03 ± 0.01 g; P > 0.05). After a 1-h recovery period, ED was performed again and was found to produce a significant decrease in fundus tone (−0.16 ± 0.02 g; P < 0.05), illustrating a partial recovery to baseline. These data are summarized in Fig. 8A. Representative experimental traces depicting the reproducible decrease in fundus tone with ED (first two traces), after bilateral NLTX microinjection (third trace), and 1 h after bilateral NLTX microinjection (fourth trace) are shown in Fig. 8B. Histological examination of the brain confirmed that the micropipette tip in each case was located in the mNTS area.

**Effect of Bilateral Microinjection of DAMGO Into the Area of the mNTS on Reflex-Induced Decreases in Fundus Tone**

Distension of the esophagus was performed in four rats with a balloon inserted into the thoracic esophagus and produced a decrease of fundus tone (−0.34 ± 0.06 g; P < 0.05). ED was performed twice with a 30-min interval between distensions. Results from distensions were similar and therefore averaged. Twenty minutes following the second ED, DAMGO (10 fmol) was bilaterally microinjected into the mNTS area. Microinjection of DAMGO decreased baseline fundus tone (−0.14 ± 0.03 g; P < 0.05). ED performed in the presence of DAMGO produced no significant decrease of fundus tone (−0.05 ± 0.03 g; P > 0.05). EDs performed 30–60 min after DAMGO were still found to produce no significant decrease in fundus tone (−0.10 ± 0.05 g; P > 0.05). These data are summarized in Fig. 8C. Representative experimental traces depicting the reproducible decrease in fundus tone with ED (first two traces), and after bilateral DAMGO microinjection (third trace) are shown in Fig. 8D.

**Effect of Intravenous NLTX on Reflex-Induced Decreases in Fundus Tone**

Distension of the esophagus was performed in seven rats with a balloon inserted into the thoracic esophagus and produced a decrease of fundus tone (−0.25 ± 0.04 g; P < 0.05). ED was performed twice with a 30-min interval between distensions. Results from distensions were similar and therefore averaged. Twenty minutes following the second ED, NLTX (10 mg/kg) was intravenously administered. NLTX had no significant effect on fundus tone but did significantly decrease MAP (25.0 ± 3.1 mmHg, P < 0.05). ED performed in the presence of NLTX produced significantly less decrease of fundus tone compared with ED performed prior to NLTX administration (−0.10 ± 0.01 g; P < 0.05). These data are summarized in Fig. 9A. Representative experimental traces depicting the changes in fundus tone with ED (left and middle traces) and after intravenous NLTX administration (right trace) are shown in Fig. 9B.

**DISCUSSION**

We tested the hypothesis that the mNTS area is a central site where activation of MORs influences gastric function by two experimental approaches. The first was microinjection of MOR agonists and antagonists into the mNTS area while gastric motility was monitored. The second was testing the effect of MOR activation and blockade on the RRR. Focusing on results from the first approach, microinjection of three MOR agonists, DAMGO, EM-1, and EM-2, into the mNTS area all decreased IGP and phasic contractions. Studies with DAMGO consisted of a wide range of doses (1 fmol to 100 pmol). The lowest doses (1–10 fmol) encompassed the EC50 for DAMGO at the MOR, which is 200 nM (54). Within this low dose range (1–10 fmol), we observed dose-dependent decreases in IGP and

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*Fig. 8: A: histograms of changes in fundus tone with esophageal distension (ED) before and after microinjection of naltrexone (NLTX, 25 pmol/30 nl) into the mNTS. *P < 0.05 paired t-test; n = 7. B: representative experimental traces depicting the change in fundus tone with esophageal distension before bilateral NLTX microinjection (1st and 2nd traces), after bilateral NLTX microinjection (3rd trace), and 1 h after bilateral NLTX microinjection (4th trace). Black bars above each trace indicate period of esophageal distension and are equal to 1 min. C: histograms of changes in fundus tone with esophageal distension before and after microinjection of DAMGO (10 fmol/30 nl) into the mNTS. *P < 0.05 paired t-test; n = 4. D: representative experimental traces depicting the change in fundus tone with esophageal distension before (1st and 2nd traces), and after bilateral DAMGO microinjection (3rd trace). Black bars above each trace indicate period of esophageal distension and are equal to 1 min.*
intravenous NLTX administration. Black bars above each trace indicate period of esophageal distension and are equal to 1 min.

Patients (13). Microinjection of opioid antagonists into the other vagovagal reflex, the gastric accommodation reflex in are consistent with the finding that naloxone counteracts an-
MORs in the mNTS also counteracted the RRR. These findings in the mNTS area did not produce significant changes in gastric motility. These findings obtained with higher doses of opioid agonists, as used in numerous earlier studies (23, 24, 29, 35, 53), are likely to exert effects located a considerable distance from the target central nervous system (CNS) site (18). In the case of our study, microinjection of higher doses of DAMGO into the mNTS area appeared to diffuse to and affect DMV neurons controlling gastric motility.

To our knowledge these data are the first to show MOR-induced effects using such low doses of opioid receptor ago-
ists in vivo and to demonstrate that activation of MORs in the mNTS area affects gastric motility. These findings obtained under the experimental conditions of anesthesia and surgical intervention are consistent with those of Glatter and Smith (15), who used an electrophysiological approach to study the effect of MOR activation on synaptic input to gastric NTS neurons identified by pseudo-rabies virus injection into the stomach. MOR stimulation in their brain slice preparation was found to suppress both inhibitory and excitatory input to NTS neurons that project to the DMV (15). Other investigators have also described the electrophysiological effects of MOR stimulation in the NTS, but these effects were not shown to occur in NTS neurons that were anatomically in the neural pathway to the stomach (3, 9, 27, 38, 39).

Whereas microinjection of MOR agonists into the mNTS area evoked robust changes in gastric function, microinjection of two opioid receptor antagonists, NLTX and CTOP, into the mNTS area did not produce significant changes in gastric motility. This finding suggests that background neuronal activity in the mNTS controlling excitability of DMV gastric-projecting neurons does not depend on MOR activation. However, a MOR antagonist does block reflex-induced decreases in gastric motility (see discussion below).

Focusing on results from the second approach, the effect of MOR activation and blockade on the RRR, blockade of MORs in the mNTS antagonized the RRR. In addition, activation of MORs in the mNTS also counteracted the RRR. These findings are consistent with the finding that naloxone counteracts another vagovagal reflex, the gastric accommodation reflex in patients (13). Microinjection of opioid antagonists into the mNTS does not alter cardiovascular reflexes (48) and does not affect the cough reflex (35). Taken together, these findings suggest that endogenous opioids in the mNTS might selectively mediate vagovagal reflexes.

Our observation that MOR activation in the mNTS area inhibits gastric tone and motility raises the question as to the mechanism(s) involved in this response. A clue as to the mechanism came from data we from our earlier study demonstrating that blockade of GABA signaling in the mNTS decreases gastric tone and motility (20), an effect that was mimicked in the present study by microinjection of MOR agonists into the mNTS. Furthermore, in situ hybridization and immunocyto-
chemical studies demonstrate that the mNTS contains the densest labeling of GABAergic interneurons in the entire NTS (10). Taken together, these findings led us to determine whether prior blockade of GABA receptors in the NTS with gabazine would prevent the inhibition of gastric motility normally seen with microinjection of DAMGO. Gabazine pretreatment did indeed completely prevent the gastric effects of MOR activation by DAMGO. The results of our in vivo studies are consistent with data from the electrophysiological studies of Smith and colleagues (14, 15) demonstrating that MOR stimulation in the mNTS acts on MORs located on cell bodies of local GABA neurons to suppress the activity of these neurons.

A concern in interpreting our data was that some of the drug pretreatments used to reveal the mechanism whereby DAMGO acts to inhibit gastric tone and motility also reduced tone and motility. Hence it was difficult to conclude whether the reduc-
tion in response to DAMGO was due to the drug pretreatment (e.g., pretreatment with the GABA receptor blocker, gabazine) or due to the decrease in tone and motility (i.e., a “floor” effect). To assess this, we tested whether a robust decrease in IGP would occur with sodium nitroprusside. The positive finding with sodium nitroprusside after gabazine indicated to us that the reduction in response to DAMGO was indeed due to removal of GABA signaling in the area of the mNTS.

It could be argued that observing a robust decrease in IGP with a peripherally administered drug (sodium nitroprusside) does not rule out the possibility that the reduction in baseline IGP with GABA receptor blockade is the reason for the loss of IGP response to microinjection of DAMGO into the mNTS. To explore this possibility further, we reviewed data obtained in our earlier study in which we microinjected L-glutamate after blockade of GABA receptors to determine whether we could note any further drop in IGP. In these studies, blockade of GABA receptors produced a per se decrease in IGP (−1.4 ±
0.23 mmHg; n = 5) that was not statistically different than the decrease observed in the present study. L-glutamate (500 pmol) microinjected at this reduced baseline IGP produced a decrease in IGP (−0.81 ± 0.09 mmHg; n = 5) that was not statistically different from the decrease in IGP produced by microinjection of L-glutamate prior to blockade of GABA_A receptors (−0.83 ± 0.17 mmHg; n = 12; Ref. 20). These data indicate that it is possible to evoke a centrally mediated decrease in IGP after GABA_A receptor blockade and suggest that the loss of the IGP response to DAMGO microinjection is due to inhibition of local GABA activity and not due to a reduction in baseline gastric tone.

On the basis of data from our previous study (20), we proposed that ongoing GABA receptor signaling overwhelms all sensory input from the GI tract. Once this ongoing mNTS GABA activity is suppressed either by GABA_A receptor antagonists (20) or MOR stimulation, glutamate released from vagal afferent terminals can excite second-order NTS neurons that in turn inhibit the DMV, producing a decrease in gastric motility. Evidence that suppression of NTS local GABA signaling enables L-glutamate released from vagal afferent terminals to excite second-order inhibitory neurons described above was obtained by demonstrating that blockade of ionotropic glutamate receptors at the NTS prevents both gabazine (20) and the MOR agonist, DAMGO, from inhibiting gastric motility.

We propose that, for the vagovagal reflex to operate, vagal afferents must release an endogenous substance that suppresses activity of NTS GABA interneurons. We suggest that the endogenous substance is an opioid that activates a MOR located on cell bodies of mNTS GABA neurons. Indeed, Scanlin et al. (42) report that activation of neurons in the nodose ganglion releases an endogenous opioid, most likely EM-2. To test this idea, we assessed whether the opioid antagonist NLTX, administered locally into the mNTS area or systemically, would suppress the RRR. Data obtained indicate that this was the case. In summary, our results demonstrate that MORs in the mNTS area not only influence gastric motility, they are also responsible for mediating the RRR.

In addition, microinjection of DAMGO into the mNTS area was found to counteract the RRR. Microinjection of DAMGO reduces intra-NTS GABA signaling before the RRR has been activated by ED. Upon activation of the RRR, there is very little GABAergic signaling to suppress, and activity in the second-order inhibitory neuron will remain relatively constant. Consistent with this explanation is our recent finding that blockade of GABA_A receptors in the mNTS with bilateral microinjection of gabazine also diminishes the RRR (unpublished data).

When we studied doses of DAMGO that were many fold higher than the dose range that corresponds to the EC_{50} value, two interesting findings were noted. First, in the dose range of 30–300 fmol, variable responses were observed that, when averaged, were not statistically significant. Second, with doses of 1 and 100 pmol, a complex triphasic response was observed, consisting of an inhibition of gastric motility that is interrupted by a transient excitatory effect. The non-effect in the dose range of 30–300 fmol may be due to opposing simultaneous actions, i.e., emerging excitatory effects of these doses neutralizing the inhibitory effects of the low doses. A question raised by the high-dose effects of DAMGO is What is responsible for the excitatory effect? One possibility is activation of a different opioid receptor. Poole and colleagues (38) report synaptic depression due to activation of a presynaptic κ-opioid receptor in NTS neurons. However, our data demonstrating blockade of the entire response to DAMGO with prior microinjection of the MOR-specific antagonist CTOP and the observation that the entire response could be obtained with a high dose of the MOR-selective agonist EM-1 indicate that only the MOR was involved. The most likely explanation is that at higher doses of DAMGO there is diffusion to adjoining areas that influence gastric function, such as the DMV. Dendrites from the contralateral DMV cross the midline and extend into the dorsal-medial area of the medulla (40). Indeed, when we microinjected the low dose of DAMGO, 10 fmol, into the DMV we observed an increase in gastric tone and motility. Microinjection of 1 pmol produced approximately a threefold greater excitatory effect. An excitatory effect of MOR stimulation in the DMV on gastric tone and motility was not unexpected. It is well documented that DMV neurons receive local GABAergic input, even from interneurons (12, 47), and MOR-mediated reduction of this local inhibition would result in disinhibition of gastric-projecting DMV neurons and excitation at the level of the stomach.

Investigators who have studied the effects of MOR stimulation in the mNTS area using end points different from gastric function (e.g., blood pressure and cough reflex) employ microinjected doses 75–100 times our 10-fmol dose (24, 31, 35). Two problems arise when such excessively high doses are microinjected into brain tissue. First is that the local injectate may activate receptors located at considerable distances from the injected area (18, 45), and the second is that local drug concentration may exceed the CNS concentration that occurs after systemic administration of the therapeutic doses. It is noteworthy that earlier studies that used the microinjection technique to locate CNS areas where the analgesic effect of opioid agonists such as morphine occur used extraordinarily high doses relative to the dose range used in the present study (1–10 fmol). For example, Yaksh et al. (53) microinjected morphine, which is similar in potency to DAMGO, in a dose of 17.5 mmol/0.5 μL. It is likely that this high dose (and injected volume) would distribute drug throughout a relatively large area of the brain, compared with our dose of 10 fmol/30 nl.

Electrophysiological studies of NTS neurons demonstrate that MOR agonists also prevent the release of L-glutamate from vagal afferent terminals (14, 15). In contrast to these in vitro brain slice studies, data from our studies indicate no evidence of this effect with the 10-fmol dose of DAMGO. The best evidence that 10 fmol DAMGO does not prevent release of glutamate from vagal sensory terminals synapsing in the mNTS area is the excitatory gastric motility response produced by blockade of ionotropic glutamate receptors following DAMGO administration. Once suppression of NTS GABA signaling was produced either by gabazine (20) or DAMGO, ionotropic glutamate receptor blockade produced a robust excitatory effect on gastric tone and motility. Inspired in large part by MOR stimulation causing a decrease of glutamate at the NTS (and at the DMV), Glazer et al. (14) predicted that MOR stimulation in the NTS should increase gastric motility, and that MOR stimulation in the DMV should decrease gastric motility. Our results indicate the opposite. Why we observe no evidence that MOR stimulation decreases glutamate release in...
an in vivo preparation, whereas a decrease in glutamate release is observed in a brain slice preparation is unclear.

In summary, we assessed actions of MOR agonists micro-injected into the mNTS on gastric function. MOR agonists decreased IGP and phasic contractions. A dose-response relationship on IGP and phasic contractions was observed at low doses, variable effects were observed at intermediate doses (30–300 fmol), and high doses (1 and 100 pmol) produced complex triphasic effects on gastric motility. MOR effects were prevented by vagotomy and pretreatment with MOR antagonists. Low-dose inhibition was mediated by suppression of local GABA signaling. Reflex inhibition of gastric tone produced by ED was inhibited by a MOR antagonist. Micro-injection of DAMGO into the mNTS also interfered with the reflex response, suggesting a physiological role for endogenous opioids in vagovagal reflex signaling. This role could relate to stimuli such as a bolus of food traveling the esophagus or gastric distension following a large meal.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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