Vagal mechanisms underlying gastric protection induced by chemical activation of raphe pallidus in rats

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Kaneko, Hiroshi, Jonathan Kaunitz, and Yvette Taché. Vagal mechanisms underlying gastric protection induced by chemical activation of raphe pallidus in rats. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1056–G1062, 1998.—Peripheral mechanisms involved in kainic acid injected into the raphe pallidus (Rpa)-induced gastric protection were investigated in urethane-anesthetized rats. Gastric mucosal blood flow (GMBF), acid secretion, and gastric injury induced by intragastric ethanol (60%) were measured in response to kainic acid (25 pg) injected into the Rpa. Kainic acid reduced ethanol-induced gastric lesions by 57%. The protective effect was blocked by vagotomy, capsaicin deafferentation, and intravenous injection of the calcitonin gene-related peptide (CGRP) antagonist CGRP-(8—37) and Nω-nitro-L-arginine methyl ester (L-NAME), but not D-arginine reversed the L-NAME action. Kainic acid injected into the Rpa, unlike outside sites, increased basal GMBF but not acid secretion. Indomethacin unmasked an acid secretory response to kainic acid. These results show that kainic acid injected into the Rpa at a dose that did not stimulate acid secretion, due to the inhibitory effect of prostaglandins, protects against ethanol-induced gastric injury through vagal-dependent activation of CGRP contained in capsaicin-sensitive afferents and nitric oxide-mediated gastric vasodilatory mechanisms.

capsaicin; calcitonin gene-related peptide antagonist; gastric mucosal blood flow; gastric acid; Nω-nitro-L-arginine methyl ester; nitric oxide; kainic acid; gastric lesions by ethanol

THE DORSAL VAGAL COMPLEX is densely innervated by thyrotropin-releasing hormone (TRH)-containing fibers that originate solely from cell bodies located in the raphe pallidus (Rpa), raphe obscurus, and the parapyramidal region (19). Convergent findings favor an important role of these medullary TRH pathways in the vagal regulation of gastric function and mucosal integrity (26, 27). Recently, we reported that activation of cell bodies in the Rpa nucleus, using kainic acid at a low dose (25 pg), protects the gastric mucosa against ethanol injury, whereas a 480-fold higher dose, stimulating gastric acid secretion, induces gastric erosions through medullary TRH-dependent pathways (10, 11, 31). The peripheral mechanisms through which low levels of Rpa cell body activation induce gastric protection are atropine and prostaglandin dependent (11).

The gastric protective mechanisms conferred by the stable TRH analog RX-77368 injected centrally at low doses stimulating gastric vagal efferent discharges (21) are associated with an increased gastric mucosal blood flow (GMBF) in the absence of stimulation of gastric acid secretion (14, 32, 33). Calcitonin gene-related peptide (CGRP) originating from capsaicin-sensitive splanchnic afferent fibers and nitric oxide (NO) play a role in the gastric hyperemic and protective responses elicited by low doses of RX-77368 (12–15, 23, 28). These observations indicate that the "efferent function" of capsaicin-sensitive splanchnic afferents releasing CGRP (7) can be activated by low levels of central vagal stimulation. Moreover, the vagal increase of gastric prostaglandin synthesis (14, 33) accounts for the lack of acid response under these conditions and also plays a role in the gastric protection through mechanisms unrelated to changes in GMBF (11, 14, 32, 33). Whether similar gastric protective mechanisms are called on by endogenous medullary TRH remains to be established. The understanding of these peripheral mechanisms can be of paramount importance as they will unravel physiological pathways through which the vagus can confer gastric protection.

In the present study, kainic acid was microinjected into the Rpa at a low dose, previously established to induce gastric protection against ethanol injury, through medullary TRH and atropine-sensitive mechanisms (11). We measured changes in gastric acid secretion and GMBF and assessed peripheral mediators involved in the gastroprotective effect against ethanol lesions, in particular the role of vagal-dependent activation of CGRP-NO pathways using vagotomy, the specific CGRP receptor antagonist CGRP-(8—37) (3), chronic capsaicin deafferentation, and NO synthase inhibitor.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan Laboratory, San Diego, CA) weighing 250–330 g were maintained ad libitum on Purina Laboratory Chow (Ralston Purina, St. Louis, MO) and tap water under conditions of controlled temperature (22–24°C) and illumination (12:12-h light-dark cycle starting at 6 AM). Rats were deprived of food for 18 h with free access to water for up to 2 h before the beginning of the study. All experiments were performed in rats anesthetized with urethane (1.5 g/kg ip; Sigma Chemical, St. Louis, MO). The body temperature was maintained at 36.5–37.5°C by a heating pad and a Yellow Springs Instrument rectal temperature probe. Experimental protocols were approved by the Animal Use Committee of the West Los Angeles Veterans Affairs Medical Center.

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Chemicals

The following substances were used: kainic acid, capsaicin, Nω-nitro-L-arginine methyl ester (L-NAME), L- or D-arginine, and indomethacin (Sigma Chemical), CGRP-(8–37) (supplied by Dr. S. St. Pierre, Dept. of Biochemistry, Univ. of Quebec, Montreal, PQ, Canada), and ethanol (Fisher Scientific, Fairmont, NJ). Kainic acid was dissolved in 0.1 M phosphate buffer (pH 7.0), capsaicin in 10% Tween 80, 10% ethanol, and 80% saline, L-NAME and L- or D-arginine in saline, CGRP-(8–37) in saline containing 0.1% BSA (Sigma Chemical), ethanol in distilled water, and indomethacin in 1.0% NaHCO₃. Solutions were prepared immediately before the experiments.

Treatments

For capsaicin treatments, rats were briefly anesthetized with ethyl ether for subcutaneous injections of capsaicin solution (12.5 mg/ml dissolved in vehicle). The total dose of capsaicin (125 mg/kg) was divided into three injections (25 mg/kg in the morning and 50 mg/kg in the afternoon on the first day, 50 mg/kg on the 2nd day). The control group received vehicle treatment (10% Tween 80, 10% ethanol, and 80% saline) under identical conditions of administration. Experiments were performed 10 days later in rats that showed the disappearance of the corneal chemosensory reflex (eye wiping for 1–3 min to a drop of 0.1% ammonium hydroxide instilled into one eye) 24 h before the study.

Microinjection into the Rpa was performed in urethane-anesthetized rats positioned on a Kopf model 900 stereotaxic instrument (Kopf Instruments, Tujunga, CA) as previously described (13). The head was fixed in a nose-down position (incisor bar set at –15 mm) and the obex region of the dorsal medulla was exposed by resecting the dorsal cervical musculature and removing the occipital skull plate and small pieces of the cerebellum. The glass micropipette (50–70 µm in diameter) was connected to a 30-cm long PE-50 polyethylene catheter, and the assembly was filled with water followed by 10 µl of either vehicle (0.1 M phosphate buffer) or kainic acid. Immediately thereafter the micropipette was positioned into the Rpa according to Paxinos and Watson's atlas (22), and vehicle or kainic acid (25 pg, 117 fmol) was microinjected in a 30-nl volume over 1 min utilizing a 1-µl Hamilton syringe assembled to the catheter. The micropipette was withdrawn after 2 min. The coordinates (in mm) used for Rpa microinjections were similar to the ones specified in our previous studies (11): Rpa 3.0 ventral from the surface of the brain, 1.5 anterior from the caudal tip of the area postrema, and 0.0 lateral from the midline. Rats were removed from the stereotaxic apparatus after the microinjection unless indicated otherwise.

Subdiaphragmatic vagotomy was performed by circular seromuscular myotomy of the esophagus at 2 cm from the gastroesophageal junction.

Gastric Measurements

GMBF. Continuous measurements of GMBF were estimated using the laser-Doppler velocimetry technique essentially as described previously (1). Under urethane anesthesia a catheter was positioned into the jugular vein to infuse saline (0.96 ml/h) throughout the experiment, and the abdomen was opened through a 3-cm midline incision to expose the stomach. A straight-tipped flow probe (P-435, Vasomedics, St. Paul, MN) was inserted into the stomach through a small incision into the forestomach. The tip of the probe was positioned on the mucosal surface at the greater curvature of the corpus by oblique contact. The abdominal incision was sutured closed. Animals were placed on the stereotaxic instrument to perform microinjection into the Rpa and maintained throughout the experiment to avoid disturbance of the gastric probe. GMBF was measured every 3 min as the voltage output of the laser-Doppler flowmetry (Laser Flo BPM 803A, Vasomedics) as previously described (1). Results are expressed as a percentage relative to the baseline levels determined during the 3-min period before microinjection of kainic acid.

Gastric acid secretion. Measurements were performed as previously described (10) in separate groups of animals. The esophagus was ligated at the cervical level, and a laparotomy was performed. The pylorus was ligated, and a double-lumen cannula was placed through a small incision into the forestomach. Gastric acid secretion was measured every 10 min by flushing the gastric lumen twice through the double-lumen gastric cannula with a 5-ml bolus of 0.9% saline at room temperature followed by one 5-ml bolus of air at the end of each 10-min period. Acid output was determined by titration (Autotitrator, Radiometer, Copenhagen, Denmark) of the flushed perfusate with 0.01 N NaOH to pH 7.0. Net acid secretion per 60 min was calculated for each rat by subtracting the average basal values of gastric acid output from each postinjection value.

Gastric erosions. Ethanol (60%, 5 ml/kg) was administered intragastrically by oral gavage using a stainless steel cannula. One hour later stomachs were removed, opened along the greater curvature, and pinned flat on cardboard. The percentage of the glandular part of the stomach containing lesions was determined by a computerized image analyzer device (MICRO/PDP-11; Digital Equipment, Maynard, MA), equipped with imaging boards (Imaging Technology, Woburn, MA) as previously described (11).

Experimental Protocols

Effect of kainic acid microinjected into Rpa on gastric acid secretion. After a 30-min period of stabilization and basal recording, indomethacin (5 mg/kg) or vehicle (1.0% NaHCO₃) was injected (1 ml/kg ip), and 60 min later rats were microinjected with kainic acid (25 pg/30 nl) or vehicle (0.1 M phosphate buffer/30 nl) into the Rpa. Gastric acid secretion was monitored every 10 min throughout the study, starting at 20 min before indomethacin or vehicle pretreatment.

Effect of kainic acid microinjected into Rpa on GMBF. After a 20- to 30-min stabilization period and a 15-min basal recording of GMBF, kainic acid (25 pg/30 nl) or vehicle (0.1 M phosphate buffer/30 nl) was microinjected into the Rpa. GMBF was monitored every 3 min for 15 min before and 60 min after microinjection of vehicle or kainic acid.

Kainic acid microinjected into Rpa-induced gastroprotection: effect of vagotomy and transmitter blockade. The following pretreatments were given before microinjection of vehicle (0.1 M phosphate buffer/30 nl) or kainic acid (25 pg/30 nl) into the Rpa: subdiaphragmatic or sham vagotomy (2 h); capsaicin (125 mg/kg sc, 10 days) or vehicle (sc, 10 days); CGRP-(8–37) (32 nmol/kg iv, 10 min) or vehicle (saline containing 0.1% BSA iv, 10 min); L-NAME (6 mg/kg iv, 10 min) or vehicle (saline iv, 10 min); L- or D-arginine (300 mg/kg iv) immediately before L-NAME (6 mg/kg iv). Single intravenous injections were performed in the jugular vein in 0.5 ml/kg and consecutive injections in 0.25 ml/kg each. The regimen of treatments was based on previous studies (13, 15). All pretreated groups were administered intragastrically with 60% ethanol 15 min after microinjection into the Rpa and gastric lesions were assessed 60 min later as previously described.
Brain Histology

At the end of each experiment, urethan-anesthetized rats were killed by decapitation, and brains were removed for fixation in a 10% Formalin, 20% sucrose solution for at least 2 days. Sections were sliced at 30 µm, mounted, and stained with toluidine blue. The locations of the microinjection sites were identified by the visualization of the point of termination of the cannula track and marked on plates reproduced from Paxinos and Watson's atlas (22).

Statistics

Results are expressed as means ± SE. A statistical analysis of time course study was performed by Statistical Analysis System general linear model procedure: repeated measured ANOVA with contrast method. Multiple group comparisons were assessed by one-way ANOVA followed by a Scheffe's test. Comparisons between two groups were performed by Student's t-test. A probability level of P < 0.05 was considered significant.

RESULTS

Effect of Kainic Acid Microinjected Into Rpa on Gastric Acid Secretion

Basal gastric acid secretion was low in urethan-anesthetized rats (2.6 ± 0.4 µmol/10 min, n = 16) and was not significantly modified by microinjection of vehicle into the Rpa (net acid secretion -1.2 ± 1.0 µmol/60 min, n = 5). Indomethacin (5 mg/kg ip) did not influence acid secretion before (Fig. 1A) or after microinjection of vehicle into the Rpa (-1.2 ± 2.2 µmol/60 min, n = 5). Kainic acid (25 pg) microinjected into the Rpa did not alter basal acid secretion in the vehicle-pretreated group (net change -0.3 ± 2.9 µmol/60 min) while stimulating acid secretion in indomethacin-pretreated rats (net increase 11.9 ± 3.1 µmol/60 min; Fig. 1A). In the indomethacin-pretreated group the peak acid response was observed 20 min after kainic acid microinjection into the Rpa, with values returning to basal levels within 30 min (Fig. 1A). When kainic acid (25 pg) was microinjected into sites nearby, but outside of the Rpa, namely into the medial (n = 1), subnucleus B medial (n = 1), or dorsal (n = 1) inferior olivary nuclei or medial lemniscus (n = 3; Fig. 1B), there was no significant increase in gastric acid secretion in indomethacin-pretreated rats (Fig. 1A).

Effect of Kainic Acid Microinjected Into Rpa on GMBF

GMBF was stable for the 15-min period before microinjection into the Rpa (Fig. 2A). GMBF was significantly increased 9 min after kainic acid (25 pg) microinjected into the Rpa and reached a plateau (137.4 ± 1.13%) between 12 and 20 min; thereafter GMBF slowly decreased, although a significant difference was observed up to 54 min after kainic acid compared with vehicle microinjection (Fig. 2A). Kainic acid microinjected into sites nearby but outside of the Rpa, namely into the gigantocellular reticular nucleus (n = 1), inferior olivary, medial nucleus (n = 1), and medial lemniscus (n = 3) (Fig. 2B), did not modify GMBF within the first 42 min postinjection, and thereafter values were decreased by 15% from basal values for the 45- to 60-min period after microinjection (Fig. 2A). Microinjection of vehicle into the Rpa did not significantly influence basal GMBF for the first 33-min period, although values were significantly decreased during the last 24-min period (78.1 ± 4.5%: Fig. 2A).

Gastric Protection Against Ethanol-Induced Lesions by Kainic Acid Into Rpa: Effect of Vagotomy and Transmitter Blockade

Oral administration of 60% ethanol produced macroscopic gastric lesions visualized as long, dark red bands linearly oriented in a cranial-to-caudal axis and primar-
Gastric lesions covered 35.2 ± 3.6% of the corpus mucosa in sham-vagotomized rats microinjected with vehicle into the Rpa. Kainic acid microinjected into the Rpa 15 min before ethanol administration significantly reduced gastric mucosal damage to 15.2 ± 1.5% of the corpus area (Fig. 3). Subdiaphragmatic vagotomy completely abolished the protective effect of kainic acid microinjected into the Rpa (Fig. 3). Pretreatment with capsaicin (125 mg/kg sc, 10 days), CGRP-(8—37) (32 nmol/kg iv, 10 min), or L-NAME (6 mg/kg iv, 10 min) also completely inhibited the gastric protective effect of kainic acid into the Rpa (Figs. 4 and 5). L-Arginine (300 mg/kg iv) injected before L-NAME restored the protective action of kainic acid, whereas D-arginine (300 mg/kg iv) had no effect (Fig. 5). Vagotomy, capsaicin, CGRP-(8—37), L-NAME, or L-arginine plus L-NAME itself did not influence 60% ethanol-induced gastric lesions in rats microinjected into the Rpa with vehicle (Figs. 3–5).

**DISCUSSION**

Kainic acid microinjected into the Rpa at a low dose significantly reduced ethanol-induced gastric mucosal lesions by 56.8%, in agreement with our previous report (11). The protective effect is site specific because microinjection of kainic acid at a similar dose into sites nearby but outside the Rpa did not result in gastric protection (11). Rpa neurons project to preganglionic neurons of the autonomic nervous system, including major efferent projections to the intermediolateral column of the spinal cord influencing sympathetic activity and more discrete projections to the dorsal vagal complex regulating parasympathetic outflow (19, 24).

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**Fig. 2.** Effect of kainic acid (25 pg/30 nl) microinjected (mi) into the Rpa on gastric mucosal blood flow in urethan-anesthetized rats. Gastric mucosal blood flow was measured as voltage output of laser-Doppler flowmetry and expressed as percentage relative to baseline levels every 3 min. Each point represents mean ± SE of 5 rats. *P < 0.05 compared with vehicle into Rpa or kainic acid outside of Rpa. #P < 0.05 compared with vehicle into Rpa. +P < 0.05 compared with respective basal value. B: diagram of coronal sections of rat brain showing schematic representation of medullary sites microinjected with kainic acid (25 pg). ●, Responsive sites to kainic acid; ▲, unresponsive sites to kainic acid. Plates adapted from Paxinos and Watson (22). Gi, gigantocellular reticular nucleus. Remaining abbreviations defined in legend for Fig. 1. Scale bar = 1 mm.

**Fig. 3.** Effect of acute truncal vagotomy on kainic acid-induced cytoprotection in urethan-anesthetized rats. Sham or subdiaphragmatic truncal vagotomy was performed 2 h before microinjection of kainic acid or vehicle into Rpa and 15 min later ethanol (60%, 5 ml/kg) was given per oral intubation to stomach. Each bar represents mean ± SE of number of rats shown at top of each bar. *P < 0.05 compared with phosphate buffer microinjected into Rpa. +P < 0.05 compared with sham operation and kainic acid microinjected into Rpa.

**Fig. 4.** Effect of systemic capsaicin pretreatment and calcitonin gene-related peptide (CGRP) antagonist on kainic acid-induced cytoprotection in urethan-anesthetized rats. Systemic capsaicin (125 mg/kg sc) or CGRP-(8—37) (32 nmol/kg iv) was given 10 days or 10 min, respectively, before microinjection of vehicle or kainic acid into Rpa. Each bar represents mean ± SE of number of rats shown at top of each column. *P < 0.05 compared with phosphate buffer injection into Rpa. +P < 0.05 compared with vehicle and kainic acid injection into Rpa.
Raphe and suggest that, in addition to the dorsal motor taglandin synthesis being enhanced by activation of together, these results are consistent with gastric pros-
gastic protective action of kainic acid microinjected into the Rpa is mediated by vagal-dependent pathways since subdiaphragmatic vagotomy abolished the kainic acid effect.

Kainic acid microinjected into the Rpa reduces ethanol-induced gastric lesions at a dose (25 pg, 117 fmol) that did not increase basal gastric acid secretion. The lack of acid response in the presence of TRH-mediated vagal cholinergic activation (Ref. 11, present results) most likely results from the antisecretory effect of vagally released gastric prostaglandins. When gastric prostaglandin synthesis was inhibited by indomethacin, kainic acid microinjected into the Rpa stimulated gastric acid secretion with a peak response that was fivefold over basal values. The acid response occurred at a dose of indomethacin that did not influence basal acid secretion, as previously reported (14, 33). Kainic acid microinjected into nearby sites outside of the Rpa did not stimulate acid secretion in indomethacin-pretreated rats showing a specificity of the response to the activation of a population of neurons in the Rpa. Other functional evidence to support an increase in gastric prostaglandin release is the demonstration that indomethacin abolished the gastric protective effect of kainic acid microinjected into the Rpa under similar conditions (11). We also previously reported that TRH analog injected into the osterna magna or dorsal vagal complex induces a vagal cholinergic release of gastric prostaglandin E2 which exerts an antisecretory effect only under low levels of vagal activation (13, 33). Taken together, these results are consistent with gastric pros-
induced gastric cholinergic release of gastric prostaglandins did not play a role (14, 28). The present

The present results also showed that vagal cholinergic-mediated gastric protection against ethanol requires the integrity of capsaicin-sensitive splanchnic afferents containing CGRP. Capsaicin pretreatment or intravenous injection of the CGRP receptor antagonist CGRP-(8—37) (3) abolished the gastric protective effect induced by kainic acid microinjected into the Rpa. Systemic capsaicin pretreatment depletes gastric CGRP contained in splanchnic afferents which provide a dense innervation of the gastric mucosal and submucosal arterioles (4, 25). Gastric protection conferred by acute activation of primary sensory neurons is mediated by the vasodilatory peptide CGRP, which is released from their terminals (7, 8). Moreover L-NAME, a peripheral NO synthase inhibitor (9), also completely abolished the gastric protective effect of kainic acid microinjected into the Rpa. The role of L-arginine-NO pathways was further established by the reversal of L-NAME action in an enantiomeric-specific manner by the coadministration of the natural substrate L-arginine. d-arginine, which is not a substrate for NO synthase (20), had no effect. Recent reports indicate that NO is an essential mediator of the gastric protective effect of peripheral CGRP contained in capsaicin-sensitive afferents (2, 17). The complete blockade of the gastric protection induced by kainic acid into the Rpa by either capsaicin pretreatment, CGRP receptor antagonism, or L-NAME is compatible with endogenous central vagal cholinergic activation recruiting the local effector function of capsaicin-sensitive afferent fibers containing CGRP (7), leading to CGRP receptor-mediated L-arginine-NO gastric vasodilatory protective mechanisms (18, 29).

Consistent with such mechanisms, kainic acid microinjected into the Rpa at a gastric protective dose produces a sustained increase in GMBF compared with a decline in GMBF over time in rats microinjected with vehicle. The enhanced GMBF was observed within 9 min and lasted for more than 50 min after microinjection into the Rpa. In contrast, kainic acid microinjected outside of the Rpa did not increase GMBF, showing the site specificity of the gastric hyperemic response. The use of laser-Doppler velocimetry has been validated in comparison with other established quantitative techniques in several studies and shown to be sensitive to rapid changes in tissue perfusion (1, 16). The decline in basal gastric blood flow occurring after 36 min of constant recording in the control group has been related to the anesthesia (16). The GMBF response induced by kainic acid into the Rpa is not secondary to changes in acid secretion (5), inasmuch as basal acid secretion was not modified at such a dose of kainic acid. We previously reported that intracisternal injection of RX-77368 at a cytoprotective dose stimulates GMBF (measured by the hydrogen gas clearance technique) through atropine, CGRP, and NO pathways, whereas prostaglandins did not play a role (14, 28). The present

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demonstration that CGRP in capsaicin-sensitive afferents and NO contribute to the gastric protection induced by kainic acid into the Rpa provides strong support for a mediation through similar neural pathways as an adequate increase in blood flow is essential for gastric mucosal tissue to withstand ethanol challenges (6, 30). In addition, the present results support a possible role of caudal raphe nuclei in the vagal regulation of GMBF. So far, very little is known about brain sites influencing GMBF (5).

In summary, kainic acid microinjected into the Rpa in a low dose increased GMBF, while not influencing gastric acid secretion, and reduced gastric erosions induced by ethanol. The lack of acid response is related to the inhibitory influence of gastric prostaglandins, and gastric protection is mediated by vagal-dependent activation of CGRP contained in capsaicin-sensitive afferents and NO pathways. The present and previous data (11) provide the first evidence that a low level of Rpa activation results in a vagal cholinergic-dependent activation of the “effenter function of capsaicin-sensitve afferents” containing CGRP. Moreover, these studies point out a possible important role of TRH-containing Rpa neurons projecting to the dorsal vagal complex in maintaining vagal cholinergic-dependent gastric prostaglandins, CGRP, and NO protective mechanisms.

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