Gastric vagal efferent inhibition evoked by intravenous CRF is unrelated to simultaneously recorded vagal afferent activity in urethane-anesthetized rats

David W. Adelson, Hovsep P. Kosoyan, Yuhua Wang, Justin Z. Steinberg and Yvette Taché

CURE: Digestive Diseases Research Center and Center for Neurovisceral Sciences & Women’s Health, Division of Digestive Diseases, David Geffen School of Medicine at the University of California, Los Angeles, Los Angeles, CA, USA 90095 and VA Greater Los Angeles Healthcare System - West Los Angeles Veterans Affairs Medical Center, Los Angeles, CA 90073

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Address for correspondence:

David W. Adelson, Ph.D., CURE: Digestive Diseases Res. Ctr., VAGLAHS – West LA VAMC, Bldg. 115, Rm. 325, 11301 Wilshire Blvd., Los Angeles, CA 90073

Ph: +1-310-478-3711 x 41961; FAX: +1-310-268-4963; E-mail: dadelson@ucla.edu
ABSTRACT

Corticotropin-releasing factor (CRF) injected peripherally or released in response to stressful challenges to the organism, reduces gastric tone and contractility, in part via vagal pathways. However, information on the changes in gastric vagal impulse activity evoked by peripheral CRF administration is entirely lacking. Using a novel ‘dual recording’ method in urethane-anesthetized rats, vagal efferent (VE) and afferent (VA) impulse activities were recorded simultaneously from separate, fine bundles dissected from the ventral gastric vagus nerve branch innervating the glandular stomach. Activity records for 38 VA single units (SUs) and 33 VE SUs were sorted from multi-unit records obtained from 13 preparations. Saline (iv) had no effect on multi-unit VE activity, while CRF (1 µg/kg, iv) immediately inhibited VE activity, reaching a nadir of 54 ± 8.0% of pre-injection levels at 3.0 min post injection. CRF (1 µg/kg, iv) inhibited 25/33 (75.8%) VE SUs and excited 3/33 (9.1%) VE SUs. In contrast to potent effects on VE activity, iv CRF did not alter multi-unit VA activity. Single-unit analysis, however, revealed 5/38 (13.1%) VA SUs excited by iv CRF at widely varying latencies (suggesting an indirect mode of action), and one inhibited VA SU. VA SUs excited following iv CRF did not respond during gastric distention and vice-versa. These experiments are the first employing simultaneous recording of gastric VA and VE units. The data demonstrate a predominantly inhibitory influence of iv CRF on VE outflow to the hindstomach, not driven by gastric vago-vagal reflex activity.

KEYWORDS: electrophysiology, visceral innervation, gastric distention, autonomic nervous system, neuroendocrine interactions
INTRODUCTION

Corticotropin-releasing factor (CRF) was the first discovered among a family of CRF-related peptides, now including urocortin 1 (Ucn 1), Ucn 2 and Ucn 3, that are involved in shaping systemic physiological responses to a variety of stressful challenges in mammals and other vertebrates (Hauger et al. 2003; Vale et al. 1981). The CRF-related peptides, as well as the two G-protein coupled receptors (CRF1 and CRF2) through which they exert their biological effects, are widely distributed both centrally and peripherally, and all are present in the gastrointestinal (GI) tract, including the stomach (Boorse and Denver 2006; Chatzaki et al. 2004a,b; Lovenberg et al. 1995; Porcher et al. 2006; Van Pett K. et al. 2000). In addition to well-characterized pleiotropic actions in the brain (Bale and Vale 2004), CRF and related peptides in the periphery have demonstrated biological effects on the gut that mimic GI effects of stress (Taché and Perdue 2004). For example, peripheral administration of CRF or related peptides inhibits gastric emptying of liquid or solid meals in several species (Asakawa et al. 1999; Lenz et al. 1988; Martinez et al. 2002; Million et al. 2002; Pappas et al. 1985; Taché and Perdue 2004), and peripheral administration of a peptide CRF2 antagonist blocks restraint stress-induced inhibition of gastric emptying in rats (Million et al. 2002). Additionally, intravenous (iv) injection of peptide CRF antagonists prevent abdominal surgical stress-induced delayed gastric emptying in rats (Barquist et al. 1992; Nozu et al. 1999), and circulating levels of CRF are elevated in humans the day following surgery (Naito et al. 1991). Together, these data suggested a role for peripheral CRF and CRF receptors in post-surgical gastric ileus, a major cause of post-surgical hospitalization days and, thus, a major healthcare cost (Luckey et al. 2003).

The mechanisms and pathways through which peripheral CRF alters gastric motor function are not yet clearly defined. CRF receptors are located at a number of sites at which
circulating or local CRF or related peptides may act to influence gastric function. These include vagal afferent (VA) fibers (Lawrence et al. 2002; Mercer et al. 1992); neurons and fibers of the antral myenteric plexus and oxyntic parietal and endocrine cells of the gastric corpus (Chatzaki et al. 2004b; Porcher et al. 2006); and the area postrema (AP) and nucleus tractus solitarius (NTS) (Van Pett K. et al. 2000), sites which lack a complete blood-brain barrier (BBB) and are, therefore, capable of sensing blood-borne signals (Gross et al. 1990, 1991). Raybould et al. (1990) demonstrated that nicotinic blockade abolished and vagotomy attenuated iv CRF-induced reductions in gastric tone and contractility in vivo, while celiac ganglionectomy or adrenergic blockade had no effect. These data indicate that the gastric inhibitory effects of iv CRF result from a combination of actions on vagal efferent (VE) and enteric nervous transmission, with sympathetic signaling playing little or no role. Since depletion of capsaicin-sensitive VA fibers did not attenuate iv CRF-evoked effects on gastric contractility and tone, it was suggested that CRF evokes changes in VE output by acting on brainstem structures via a humoral route (Raybould et al. 1990). However, a role for VA signaling in mediating changes in VE output evoked by iv CRF cannot be excluded on the basis of these data, because a large population of gastric VA fibers, including a majority innervating intraganglionic laminar endings or intramuscular arrays or endings, survive capsaicin pretreatment and retain the capacity to signal gastric distention to the NTS (Berthoud et al. 1997). Further, the presence of CRF receptors on VA fibers suggests some role for CRF peptides in modulating VA discharge (Lawrence et al. 2002; Mercer et al. 1992).

To date, no published reports exist detailing either VA or VE activity evoked by peripheral injection of CRF, and some controversy exists regarding the influence of central administration of CRF on vagal outflow. Kosoyan et al. (1999) initially reported an inhibitory
effect of CRF injected intracisternally (ic) on gastric VE output. In contrast, Lewis et al. (2002) demonstrated a nearly exclusively excitatory effect of CRF superfusion on gastric-projecting dorsal motor nucleus (DMN) cells in an in vitro brainstem slice preparation. On the basis of those findings and results from in vivo physiological studies using microinjection of CRF into the dorsal vagal complex (DVC), Lewis et al. emphasized CRF-evoked excitation of the VE nonadrenergic noncholinergic inhibitory DMN pathway in producing gastric inhibition, and questioned the role of CRF-evoked inhibition of the VE excitatory pathway in the response (Lewis et al. 2002). Due to these contrasting results on the central action of CRF in modulating gastric VE activity, and because the pathways and biological responses recruited by peripheral vs. central injection of peptides, including CRF, may differ (Nakade et al. 2003; Taché et al. 2006; Yokohama et al. 1999), we deemed it important to directly determine the effects of iv injection of CRF on gastric vagal activity.

The present study was therefore designed to directly examine the responses of VE and VA single-units (SU) in the subdiaphragmatic ventral gastric vagus nerve (VGV) to iv CRF injection, and to determine the presence of temporal relationships, if any, between gastric VA activity from and VE outflow to the stomach evoked by this peptide. To accomplish these aims, we adapted classical techniques for unit recording from visceral nerves in vivo to create a novel ‘dual recording’ method allowing simultaneous monitoring of efferent and afferent unit activity in separate strands of the VGV in individual urethane-anesthetized rats.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (250-350 g, Harlan Laboratories, San Diego, CA) were housed under controlled conditions of temperature (22-24°C) and illumination (12 h light cycle, starting at 6:00 AM) and maintained with Purina Chow and tap water *ad libitum*. Rats were deprived of food overnight (>14 h) but allowed free access to tap water up to the beginning of the experiments. Rats were anesthetized by intramuscular injection of 25% urethane (1.5 ml/kg) into the right hindlimb, followed by additional doses of up to 0.2 ml 25% urethane intraperitoneally, if needed, to render the animal areflexic to strong ear- or tail-pinch prior to initiating surgery. Protocols were approved by the Veterans Administration Institutional Animal Care and Use Committee (ACORP 05-058-02).

**Chemicals**

Rat/human CRF (r/hCRF; Peptide Biology Laboratories, Salk Institute, La Jolla, CA, USA) was kept in powder form at −80°C and dissolved in saline immediately before injection. CCK-8 (Sigma, St. Louis, MO) stored in aliquots (100 μg/μl, dissolved in saline and 0.1% bovine serum albumin, BSA) at −80°C was diluted in saline + 0.1% BSA just prior to injection. Peptides or vehicle were injected iv in a volume of 0.1 ml, followed by a 0.05 ml saline flush. All other chemicals used were purchased from Sigma.

**Surgery**

Animals were tracheally cannulated to insure airway patency, and maintained at 35.5-37.5°C on a feedback-controlled heating block. Rectal temperature was monitored continuously via a thermistor probe and thermometer with analog output (YSI, Inc., Dayton, OH). An iv cannula was inserted into the left jugular vein to allow continuous infusion of 0.4 ml/h sterile, pyrogen-free 0.9% saline (Sigma) to maintain hydration, and for iv injection of peptides. EKG
electrodes were attached to fore and hind paws and connected to a differential amplifier (Model 1700, A-M Systems, Carlsborg WA) to allow recording of heart rate. Animals were judged to be in good condition if their heart rate prior to CRF injection was within the normal physiologic range for the rat of 250-450 beats per min (bpm) (Harkness and Wagner 1989). Typically, heart rate was in the range of 350-380 bpm prior to laparotomy. Laparotomy was performed, and the skin flaps were secured to a stainless steel ring, exposing the abdominal viscera. All exposed surfaces were kept moist by covering with cotton soaked in warm 0.1 M phosphate-buffered (pH 7.4) saline (Sigma). The duodenum was ligated at a point 1-2 cm distal to the pylorus, and an incision was made in the duodenum proximal to the ligation. A polyethylene cannula (PE-240, Becton Dickson, Inc., Franklin Lakes, NJ) was inserted into this incision, passed into the stomach via the pylorus, and secured by a ligature at its point of duodenal insertion. This transpyloric cannula was used to inject 0.5 ml of saline into the stomach to maintain a slightly distended state for the duration of the experiment, and for injecting fluid for gastric distentions.

As it approaches the stomach, the VGV bifurcates into a rightward branch, which courses toward the lesser curvature, and a leftward branch (the anterior fundic branch), which courses toward the greater curvature (Legros and Griffith 1969; Prechtl and Powley 1985). Recordings were made in the former of these two branches, distal to the branch point. None of the recordings reported in this study were made in the anterior fundic branch. Approximately 1 cm rostral to the gastroesophageal junction, connective tissue anchoring the VGV to the esophagus was dissected away, and a segment of the right branch of the VGV was placed on a dissecting platform immediately above the esophagus. Using a dissecting microscope (Olympus SZ30) for observation and fine forceps, a "picking window" was opened in the epineurial and perineurial sheaths from the exposed surface of the VGV for a length of 2-5 mm, and the abdominal cavity
was filled with warm mineral oil to provide electrical isolation for the recording. A quadripolar platinum wire electrode (wire diameter 25 μm, A-M Systems, Carlsborg, WA) comprising two separate bipolar electrodes was positioned above the picking window. Two separate twigs of the VGV, each approximately 10-20 μm in diameter, were gently teased from the nerve, and each was placed on one pole of one of the pair of bipolar electrodes. One was picked (severed) at the caudal margin of the picking window and teased free toward the rostral margin and, thus, only centrifugal (efferent) impulse activity was recorded from this filament. The other was picked (severed) from the rostral margin of the picking window and teased toward the caudal margin and, thus, only centripetal (afferent) activity was recorded from this filament. Connective tissue was attached to the indifferent pole of each bipolar electrode. This arrangement allowed simultaneous recording of VE and VA activity in separate filaments of the VGV. Filaments were picked and placed on the electrodes until both an “efferent” and an “afferent” filament were isolated, each containing few enough units with distinctive waveforms adequate to allow reliable sorting of the activity into SU records. Once appropriate filaments were isolated, the preparation was left undisturbed for at least a half hour, until VE activity, VA activity, and gastric tone and motility had stabilized. Fig. 1 illustrates the use of this method to directly observe the vago-vagal reflex evoked by bolus iv CCK-8 injection (3 μg/kg).

**Intragastric pressure recording**

In initial experiments, intraluminal gastric pressure (IGP) was measured using a balloon-tipped catheter inserted into the stomach via a forestomach incision and connected to an external pressure transducer (BLPR, WPI Inc, Boca Raton, FL). The tip of the cannula was positioned at the distal end of the corpus and the incision was closed around the cannula using silk sutures. In
later experiments, IGP was measured using a Millar MIKRO-TIP catheter pressure transducer (SPR-524, Millar Instruments, Houston, TX) inserted into the distal corpus via a similar forestomach incision, and connected to a transducer control unit (TCB-600, Millar Instruments). Pressure signals were amplified using a TBM4 (World Precision Instruments) transducer amplifier.

*Electrophysiological recording*

Each bipolar electrode was connected to a separate channel of a multi-channel differential amplifier (Model 1700, A-M Systems, Carlsborg WA) to allow separate recording from the two nerve twigs. Impulse activity from each bipolar electrode was amplified 1,000-fold and the signal was filtered with a passband of 100 Hz to 5 kHz. Impulse activity, along with all physiological data (rectal temperature, EKG, and IGP), were acquired using a Micro1401 A/D interface (CED, Ltd., Cambridge) connected to a PC computer running Spike 2 (CED, Ltd., Cambridge) data acquisition software. Stimulus application times were marked with a foot pedal on a separate channel.

*Sorting of unit waveforms*

VE and VA activities were recorded on separate channels and multi-unit activity on each channel was sorted into SU activity using Spike 2 software. Templates were formed by setting initial template widths to 12% of spike amplitude, and requiring matching of a minimum of 60 to 65% of points within the template envelope. The full file was scanned, allowing as many templates as needed to be formed and combining no templates. Units were then assigned marker codes based on these templates, spikes corresponding to each marker code were reviewed, and
similar waveforms were combined under single marker codes until a minimum number of
distinct marker assignments remained. In the event that some markers clearly contained more
than one spike shape, subsorting was performed. Units whose waveforms could be reliably
distinguished from all other waveforms in the record were registered as SUs.

Data analysis

Measurements of impulse activity levels and IGP were made using proprietary software
scripts developed by us for the purpose, using the Spike 2 scripting language. Percent change in
activity for each stimulus was calculated by subtracting the mean rate of discharge during the 5
min preceding the injection (pre-stimulus mean) from the mean rate during the 5 min following
the injection, and dividing by the pre-stimulus mean. Five-min peak (or nadir) percent changes
following iv injections were calculated by normalizing the mean discharge rate over the 5-min
interval during which discharge was maximal (or minimal) to the pre-stimulus mean. Percent
change in phasic IGP activity was obtained by first removing the respiratory component of
phasic pressure changes by smoothing the IGP trace with a 1-s time constant, removing the DC
component of the IGP trace (time constant 30 s), and taking the root mean square (RMS)
amplitude of the resultant trace using Channel Process facilities provided in the Spike 2 software
(CED Ltd., Cambridge). For time course plots, the mean discharge rate during each 30-s interval
was normalized to the pre-stimulus mean. For distentions, the percent change in activity was
determined by normalizing the mean discharge rate during the distention to the pre-stimulus
mean over an equivalent period (typically 30 s).

Experimental design
Following the initial 30-min rest period, responses to 2-ml gastric distention for 30-45 s were recorded. In two experiments, distilled water, rather than saline, was used to distend the stomach. This relatively small distention volume was chosen to avoid disturbing the recording conditions. A minimum of 15 min after testing responses to distention, 0.1 ml of saline was injected iv, followed 15 min later by iv CRF injection (1 μg/kg). Responses to iv CRF injection were recorded for a minimum of 30 min after injection. The dose of CRF was selected based on our previous dose-response studies showing a vagal-dependent decrease in IGP in rats when injected iv at 0.05-5 μg/kg (2.1-210 pmol), and corresponded to 20% of the dose yielding the maximal observed decrease in IGP in urethane-anesthetized rats (Raybould et al. 1990). Saline iv injection did not increase heart rate significantly (1.1 ± 1.5 bpm at 5 min post injection). Heart rate in the 5 min prior to CRF injection was 427.5 ± 15.6 bpm (n = 7), and it rose significantly (P < 0.02, paired t-test) by 10.3 ± 2.6 bpm by 5 min post-injection.

At the end of the experiment, the stomach was probed with a camel’s hair brush and/or von Frey hair type stimulators to locate the receptive fields of VA units present within the filament. All “afferent” filaments contained VA receptive fields located in corpus, antrum, and/or pylorus, but no receptive fields were identified in the non-glandular forestomach, consistent with the recording site being distal to the divergence of the anterior fundic branch of the VGV. After receptive field determination, the central source of the activity in the “efferent” filament (i.e., the filament from which activity was recorded from the central cut end) was verified by cutting the VGV proximally to eliminate centrifugal (efferent) activity originating centrally. Next, the peripheral source of activity in the “afferent” filament (i.e., the filament from which activity was recorded from the peripheral cut end) was verified by cutting the VGV distally to eliminate centripetal (afferent) activity originating peripherally (Fig. 2).
Statistics

All values reported are means ± SEM. Differences between pre and post injection discharge rates were analyzed by comparing discharge per 2.5 min bin post injection to each of the two 2.5-min pre-injection control intervals using RM ANOVA with Dunnett’s post-test. Differences in initial discharge rates between different sub-populations of units were tested for significance using ANOVA. All statistical tests were performed using the software program Prism version 3.0 (GraphPad Software, San Diego, CA).

RESULTS

Recordings of responses to iv CRF were obtained from 13 preparations. In 8 of these, both VE and VA activity were recorded, while in 5, only VA activity was recorded. Activity records for a total of 38 VA SUs and 33 VE SUs were generated. IGP was simultaneously monitored in 10 preparations. In 6 of these, phasic contractile activity was well developed.

Intravenous saline injection did not alter multi-unit discharge in either VE or VA bundles in urethane-anesthetized rats (Fig. 3A). CRF (1 µg/kg, iv) also did not alter multi-unit activity in VA bundles; however, it caused a marked drop in multi-unit VE activity beginning within 10-20 s of starting the injection. Multi-unit VE activity reached a nadir of 54 ± 8.0% of pre-injection levels at 3.0 min post injection (Fig. 3B), and remained significantly reduced relative to pre-injection levels for 20 min post injection ($P < 0.05, n = 8$). CRF iv caused a drop in IGP over the 5 min post injection relative to saline controls in 5 of 10 preparations (−17.8 ± 8.4% vs. 0.6 ± 1.4%), but as a group no statistically significant reduction occurred over this interval due to the absence of a reduction in the other 5 preparations. VE discharge inhibition evoked by CRF in
preparations in which a drop in IGP was absent did not differ significantly from those in which it was present. In preparations in which phasic contractile activity was present, CRF iv significantly reduced the amplitude of this activity relative to saline iv injection (−43 ± 8.3% vs. 2.5 ± 8.0%, \(P < 0.05, n = 6\)). VED inhibition evoked by iv CRF in preparations lacking phasic activity did not differ significantly from those in which it was present. A comparison of the VE discharge with the IGP in individual preparations revealed that the decrease in VGV efferent activity preceded by several seconds iv CRF-induced drops in intragastric tone and inhibition of rhythmic gastric contractions where present (Fig. 3C).

**SU analysis of VGV efferent fiber responses to iv CRF and gastric distention**

In response to iv CRF (1 µg/kg), the ongoing activity in 25/33 (75.8%) VE SUs was significantly inhibited (20% or more) relative to pre-injection discharge rates (Fig. 4Ai, iii, Biii), while in 5/33 (14.2%) VE units, there was less than a 15% change (increase or decrease) relative to pre-injection values (Fig. 4Aii, Bii), and in 3/33 fibers (9.1%), CRF evoked an increase in impulse activity (>20% increase in discharge rate; Fig. 4Bi). There was no significant difference in the initial discharge rates of VE units inhibited (2.6 ± 0.4 impulse/s), unaffected (2.9 ± 1.1 impulses/s) or excited by iv CRF (3.3 ± 0.6 impulses/s).

Distention of the stomach with 2 ml fluid caused a peak increase in IGP of 12.4 ± 2.3 cm H₂O, and a mean increase in IGP over the 30-45 s of distention of 5.6 ± 1.0 cm H₂O (\(n = 8\)). Prevailing phasic activity, present in 6 of 10 preparations in which pressure was recorded, was inhibited or abolished during the distention. Responses to gastric distention were determined for 20 efferent units for which responses to iv CRF were also recorded. Of these, 4/20 (20%) were inhibited by 2-ml distention by more than 20% relative to pre-stimulus control levels (−31 ±
5.4%, 6/20 (30%) were excited by distention (83.3 ± 27.7%, range 33.9-129%), and 10/20 (50%) altered their discharge by less than 20% relative to pre-stimulus control levels. All VE units inhibited by distention, 9/10 (90%) unaffected by distention, and 3/6 (50%) units excited by distention, were inhibited by iv CRF. Of the remaining 3/6 units excited by distention, one responded to iv CRF with increased discharge.

**SU analysis of VGV afferent fiber responses to iv CRF and gastric distention**

CRF (1 µg/kg, iv) injection did not significantly alter multi-unit activity in VA bundles, and this was reflected by the lack of response in 32/38 (84.2%) VA SUs (e.g., Fig. 5A). However, SU analysis also revealed that 5/38 (13.1%) VA SUs responded following iv CRF, but not iv saline, with increased activity organized in a distinctive pattern, with frequent pairs or triplets of impulses (Fig. 5B,C). The most active of these units (Fig. 5C) discharged at a rate of 1.1 Hz prior to iv CRF, while the other 4 units had pre-CRF discharge rates of 0.02 ± 0.01 Hz. Discharge rates among the 5 SUs excited by iv CRF rose to peak levels of 1.9 ± 0.4 Hz after 6.8 ± 1.5 min post-stimulus. Activity records during distention were obtained for 3/5 CRF-responsive VA units, none of which responded. One of 38 VA units (2.7%) was inhibited by iv CRF. Discharge in this unit was organized into clusters of activity with a period of ~70 s prior to CRF (Fig. 5D), clearly distinguishable from the ~12-s period of gastric contractions at the pacemaker frequency. No significant discernible increase in multi-unit VA activity was observed because the peak discharge rate of the fibers responding to iv CRF was low compared to the ongoing activities of other fibers, and the response latencies were variable, so that the peaks in activity of responsive units did not coincide.
Distention responses were recorded in 33 VA fibers. Of these, 11/33 fibers (33.3%) gave no response during 2-ml gastric distention, while 6/33 responded with only one or several impulses during distention. Responses evoked during distention in the remaining fibers fell into one of two types, as illustrated in Fig. 6. Seven distention-responsive fibers responded during distention with stimulus-related mechanoreceptive responses. In these units, peak discharge occurred concomitantly with the rising edge of IGP. Six of these units accommodated to varying degrees during the course of the distention, with discharge levels dropping on cessation of the stimulus (Fig. 6A,B), while one exhibited elevated discharge only during the rising edge of distention. In contrast, the temporal pattern of activity in 9 other VA fibers did not correspond to the distention stimulus. These units increased their activity beginning several seconds after the onset of distention, with the evoked discharge rate decaying slowly over a long period following cessation of distention, often remaining at an elevated level persistently thereafter (Fig. 6C,D).

The sole VA unit inhibited by iv CRF was among this latter type of distention-responsive fibers. Six of the nine units exhibiting this type of response to distention were found in the two preparations in which distilled water, rather than saline, was used to distend the stomach. A number of fibers in this delayed distention-responsive group had discharge organized into bursts (Fig. 6C) or intermittent pairs or triplets of impulses, often with the second discharge in a pair occurring at or near a consistent, apparently preferred interspike interval, leading to the appearance in the instantaneous frequency plot illustrated in Fig. 6D.

Relationship of VGV afferent to efferent activity changes evoked by iv CRF

Fig. 3 compares the aggregate responses to iv CRF (1 µg/kg) of all efferent and afferent multi-unit bundles, demonstrating that the drop in multi-unit VE discharge was not accompanied
by a change in multi-unit VA discharge in the VGV at the recording site, a point located distal to
the divergence of the anterior fundic branch. However, since a small subset of VA fibers was
excited by iv CRF, and one fiber was inhibited by iv CRF, the latency to excitation (or
inhibition) in each of these units was compared to changes in VE activity recorded in the same
preparation. In no preparation were iv CRF-evoked changes in VA activity observed prior to
changes in VE activity recorded simultaneously. The shortest response latency among the 5 VA
fibers excited by iv CRF is illustrated in Fig. 7A. In this unit, the onset of excitation
corresponded very closely to the onset of inhibition of VE activity. Fig. 7B illustrates the activity
of another unit excited by iv CRF showing a similar pattern of excitation to that seen in Fig. 7A,
but occurring at a latency of more than 5 min post injection, long after the maximal inhibition of
VE activity, suggesting that the VA excitation in this unit was not linked to iv CRF effects on
VE. Inhibition of activity in the sole VA unit inhibited by iv CRF (Fig. 7C) lagged the inhibition
in VE activity recorded simultaneously by more than 40 s.

DISCUSSION

This study is the first to investigate impulse activity evoked in the subdiaphragmatic
vagus nerve by iv CRF. A novel ‘dual recording’ technique was used to record VA and VE
activity simultaneously in separate centrally-cut (“afferent”) and peripherally-cut (“efferent”)
filaments teased from the VGV trunk. The method allows direct monitoring of temporal
relationships between VA and VE activity in individual preparations. Recordings were made in
the VGV distal to the point at which the anterior fundic branch, which supplies the forestomach,
diverges from it (Legros and Griffith 1969; Prechtl and Powley 1985). The rat VGV innervates
the stomach, pylorus, proximal duodenum, and pancreas (Berthoud et al. 1991). Consistent with
the gross anatomical pathway of the VGV distal to the branching of the anterior fundic branch, VA receptive fields were found in the corpus, cardia region, and antrum, but not in the non-glandular forestomach. At this recording site, iv CRF powerfully and rapidly inhibited ongoing activity in a majority (75.8%) of VGV VE fibers. CRF iv also caused a drop in IGP similar to that previously reported (Raybould et al. 1990) in half of the preparations tested, but as a group, the drop in tone over 5 min post injection was not significantly different from saline controls. This was attributed to differences in the state of the stomach between preparations, since VE inhibition did not differ in preparations lacking an IGP response. Phasic gastric contractile activity was present in 60% of preparations, equivalent to the proportion noted by Andrews and Scratcherd in a study of vagal control of gastric motility in the ferret (Andrews and Scratcherd 1980). Where phasic activity was present, iv CRF significantly inhibited it. VE inhibition preceded by several seconds the inhibition of both tonic and phasic IGP activity where present (Fig. 3).

VE inhibition evoked by iv CRF occurred without a concomitant change in mass VA activity, and well before any response in 4 of the 5/38 VA SUs excited following iv CRF. The vagal mechanisms involved in the iv CRF-evoked drop in intragastric tone and contractions thus differ clearly from the vago-vagal reflex responsible for gastric accommodation in response to either iv CCK-8 injection, or endogenous release of CCK-58 postprandially (Glatzle et al. 2003; Raybould et al. 1986; Reeve, Jr. et al. 2003). Fig. 1 illustrates the first simultaneous recording of both limbs of the vago-vagal reflex activity evoked by iv CCK-8 injection, demonstrating the concomitant activation of VA and inhibition of VE activity in the VGV. The present data support the hypothesis of a humorally-mediated influence of circulating CRF in driving the response
(Raybould et al. 1990). The involvement of humoral pathway in vagally-mediated effects of iv CRF on colonic motility has also recently been established (Tsukamoto et al. 2006).

In the present study, 75.8% of VE fibers in the VGV were inhibited by 1 µg/kg CRF while only 9.1% were excited, with the rest unaffected. Most VE fibers in the VGV originate from cell bodies in the DMN (Fox and Powley 1985; Norgren and Smith 1988). The great majority of gastric-projecting DMN cells are cholinergic (Armstrong et al. 1990; Ferreira et al. 2001; Sahibzada et al. 2002), comprising both gastric excitatory and gastric inhibitory subsets (Andrews and Scratcherd 1980). Fewer than 10% of GI-projecting DMN cells are dopaminergic, the majority of these being also cholinergic (Armstrong et al. 1990; Guo et al. 2001; Tayo and Williams 1988; Tsukamoto et al. 2005). Less than 5% of the gastric DMN projection in the VGV is nitrergic (Hyland et al. 2001; Zheng et al. 1999). An estimated 10% of descending impulse activity in the VGV originates from subcervical sources (Wei et al. 1992), which include vagal paraganglion cells (Prechtl and Powley 1985, 1990), descending axon collaterals of afferent fibers (Wei et al. 1992, 1995), and a small number of catecholaminergic sympathetic efferent fibers (Yang et al. 1999). Given the large proportion of fibers inhibited by iv CRF, coupled with the predominance of cholinergic vagal motor fibers, it seems likely that the great majority of VE fibers inhibited by iv CRF comprised cholinergic neurons of the excitatory pathway to the hindstomach. This is consistent with the observation that the drop in VE activity mirrored and preceded the inhibition of gastric rhythmic activity at the pacemaker frequency, as the amplitude of such contractions is controlled by vagal cholinergic drive (Andrews and Scratcherd 1980). The minority of VE fibers (3/33) excited by iv CRF may be part of the gastric inhibitory VE pathway, but it is unlikely that they were all nitrergic, as nitrergic cells comprise less than 1% of the DMN projection to the gastric corpus and antrum in the rat (Zheng et al. 1999). Since the
functions of the rat forestomach and hindstomach differ, the innervation and proportion of fiber types in the anterior fundic branch likely differ from those recorded in the present study.

The predominant inhibitory effect of iv CRF on VGV VE fibers is similar to that observed by Kosoyan et al. (1999) in response to ic injection of CRF, possibly indicating similar sites of action. Although circulating CRF does not cross the BBB (Martins et al. 1996), it may act on CRF receptor-bearing neurons located in regions of the DVC, including the AP and NTS (Van Pett K. et al. 2000), that are invested with capillaries lacking a complete BBB. These brainstem nuclei are sites where circulating concentrations of a variety of hormones and other solutes can be directly detected by central neurons (Gross et al. 1991; Gross et al. 1990).

In contrast to the inhibitory effects of iv or ic CRF on VGV VE activity observed in vivo in the present study and that of Kosoyan et al. (1999), superfusion of 100 nM CRF in an in vitro rat pup brainstem slice preparation yielded almost exclusively excitatory responses from gastric-projecting DMN cells (Lewis et al. 2002). In the same study, microinjection of CRF (20 nl of a $10^{-6}$ M solution) into the DVC of adult rats reduced bethanechol-stimulated motility of the gastric corpus, and this inhibitory effect was abolished by vagotomy or iv L-NAME. Those data suggested that the inhibitory actions of iv CRF on gastric motility resulted from CRF-evoked activation of vagal inhibitory non-adrenergic non-cholinergic efferent pathways. Those data do not, however, exclude a role for the inhibition of excitatory VE drive to the stomach in response to iv CRF, since strong cholinergic stimulation of gastric contractile activity by systemic bethanechol would likely have obscured any effect of withdrawal of excitatory VE cholinergic tone on gastric motility. The reasons for such markedly different proportions of excitatory and inhibitory responses evoked by CRF in DMN cells of the brainstem slice preparation compared to VGV VE responses in the in vivo preparations remain obscure. However, a similar difference
exists between the excitatory responses of gastric-projecting DMN neurons to CCK-8 superfusion in the brainstem slice preparation and the inhibitory responses in VE fibers following iv CCK-8 injection in vivo (Bucinskaite et al. 2000; Zheng et al. 2005). One possibility is that observed differences in neural responses result from differences in the areas or structures within the DVC to which the peptides have access in the two preparations. Peptides administered iv or ic in vivo may only access specialized sites within the DVC, while peptides micro-injected in vivo or superfused in vitro likely access the entirety of the neuronal cell membranes. Alternatively, differences between responses obtained in the in vitro slice vs. the in vivo preparations may result from differences in the neuronal connectivity extant in each, or from differences in the developmental stage of the cells being studied.

In contrast to the large proportion of VE fibers immediately responsive to iv CRF injection, only 5/38 VA fibers in the VGV were excited following iv CRF, while one VA fiber was inhibited. The long response latencies in 4 of the 5 units excited suggest that CRF may act on such fibers indirectly. For example, iv CRF might evoke mast cell degranulation, thereby releasing mediators capable of exciting VA fibers (Castagliuolo et al. 1996). The variability in the latency to response, then, might be due to variability in the distance between the site of CRF-evoked mediator release and the VA receptive field, possibly explaining the similarity in response pattern, despite wide differences in response latency.

Among VA units sampled, we observed two types of units responsive to 2-ml gastric distention. The first comprised mechanoreceptive units whose activity was directly related to the temporal profile of gastric distention, corresponding to those described by a number of groups in rats and ferrets (Andrews et al. 1980; Blackshaw et al. 1987; Davison and Clarke 1988). The second type were units excited at a variable latency following the initiation of distention, whose
excitation continued long after the distention ended. Activity in these units resembled an illustrated example of that evoked in a VA antral mucosal chemoreceptor by a drop of distilled water applied to the afferent receptive field (Clarke and Davison 1978). The sole VA unit inhibited following iv CRF injection was of this latter type. Six of the nine units with this type of response to distention were from preparations in which distilled water rather than saline was used to distend the stomach, suggesting that the response might be driven by changes in intraluminal tonicity. None of the VA units exhibiting either type of response to distention were excited by iv CRF.

The rarity or absence of direct VA responses to iv CRF, at a dose 20-fold greater than the minimum dose observed to evoke significant changes in gastric motility (Raybould et al. 1990), begs the question of the function of CRF receptors on subdiaphragmatic VA fibers (Lawrence et al. 2002; Mercer et al. 1992). One possibility is that the dose of circulating CRF used in these experiments is too low to excite VA fibers directly, but that higher concentrations occurring locally following release of CRF or related peptides from cells in the immediate terminal microenvironment might be capable of activating VA fibers directly. For example, CRF released in the periphery at inflammatory sites does not appear in the circulation in measurable quantities, although it is markedly elevated at inflammatory sites (Karalis et al. 1991). Enteric neurons, endocrine cells, parietal cells, and infiltrating inflammatory cells all contain CRF or related peptides (Chatzaki et al. 2004b; Porcher et al. 2006; van Tol et al. 1996). To explore whether high local concentrations of CRF agonists might activate afferents, we have recently used an in vitro stomach-vagus nerve preparation to test the responses of VA fibers to close arterial injection of the CRF2 selective agonist Ucn 2, in the dose range of 25-1200 ng (Wang et al. 2006). Given the close arterial injection and small volume of the gastric vascular network
relative to the full volume of the circulation *in vivo*, the concentrations of ligand in the gastric capillaries *in vitro* were much higher than those in the *in vivo* experiments performed here. While a larger proportion of gastric VA fibers responded *in vitro*, the response to repeated injections was not dose-related, and what response did occur was much weaker than, and typically delayed relative to, CCK-8 responses measured as a control in the same experiments (Wang et al. 2006). These data also suggest the possibility of an indirect excitatory path of action.

A second possibility, which we favor, is that CRF receptors on VA fibers do not directly mediate impulse initiation, but instead modulate the responses of VA fibers to other mechanical and/or chemical stimuli. One intriguing possibility for the function of CRF receptors on VA fibers is suggested by the failure of VA distention-sensitive units identified in this study to reduce their discharge in response to iv CRF injection, even in those preparations in which IGP reductions were apparent. While it is possible that the threshold for response of these mechanoreceptors was simply lower than the changes evoked by iv CRF injection under the conditions of these experiments, it is also possible that CRF receptors on such fibers may uncouple the mechanosensitive and the impulse-generating functions of the VA terminal. Further work will be necessary to investigate these alternative hypotheses.

In summary, we have developed a new approach permitting the recording of VE and VA SU impulse activity simultaneously, and have used the method to obtain the first recordings of VE and VA responses to peripheral CRF injection. The results indicate a rapid, predominantly VE inhibitory effect of iv CRF which precedes the associated fall in intragastric tone and contractility and is independent of VA input, and a delayed, likely indirect, effect of circulating CRF on the activity of a distinctive minority of VA fibers. These data may be relevant to understanding the vagal mechanisms involved in the inhibition of gastric motor function during
sustained stress or following surgery, conditions associated with elevated plasma levels of CRF (Luckey et al. 2003; Nishioka et al. 1994; Nishioka et al. 1993; Raybould et al. 1990; Taché and Perdue 2004).

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REFERENCES


FIGURE LEGENDS

Fig. 1 – Dual recording used to directly observe a CCK-8-evoked vago-vagal reflex. The upper plot illustrates afferent excitation in a single VA fiber in the VGV in response to iv injection of CCK-8 (3 µg/kg). The central trace shows the accompanying drop in IGP. The lower plot illustrates multi-unit efferent discharge depressed by iv CCK-8. All three traces were recorded simultaneously.

Fig. 2 – Receptive field probing and use of serial vagotomy to verify afferent and efferent identity of recorded fibers. Upper trace: multi-unit afferent discharge; Lower trace: multi-unit efferent discharge recorded simultaneously. Underscores in upper trace (afferent record) indicate mechanical probing in (1) fundus, (2) cardia region, and (3) antrum. This probing did not alter activity in the efferent bundle (lower trace). Afferent activity was abolished following section of the VGV immediately distal to the recording site (distal Vx), while efferent activity was abolished following section of the VGV immediately proximal to the recording site (proximal Vx).

Fig. 3 – Multi-unit efferent and afferent responses to iv CRF. (A) Saline (iv) injection did not alter multi-unit VA or VE activity. (B) CRF iv injection caused an immediate, significant decrease in multi-unit VE discharge (n = 8), but no significant change in multi-unit VA activity. VE discharge remained significantly (P < 0.05) depressed below control levels for 20 min. (C) Example of the temporal relationship between multi-unit VA discharge (VAD, upper trace), VE
discharge (VED, center trace) and IGP (lower trace). VED inhibition preceded the onset of iv CRF-evoked reductions in tonic and phasic IGP.

**Fig. 4 – Single-unit efferent responses to iv CRF.** Panels A and B show histograms of activity in three separate SUs recorded simultaneously in each of two separate preparations. A(i), A(iii), and B(iii) show similar, pronounced and long lasting iv CRF-evoked inhibition of activity. B(ii) exhibited a less pronounced and shorter-lived inhibition, than that in B(iii). Discharge in A(ii) was unaffected by iv CRF, while that in B(i) was excited by iv CRF. Insets show superimposed impulse waveforms demonstrating consistent unique shape of each spike.

**Fig. 5 – Single-unit afferent responses to iv CRF.** In all panels, the instantaneous frequency plot of the activity evoked by iv CRF is shown above a histogram illustrating the same activity. (A) An example of the majority of VA units unresponsive to iv CRF. (B-D) A minority of VA SUs responded to iv CRF (1 µg/kg). (B,C) Units responding to iv CRF with an increase in firing rate. Impulse activity in these units frequently occurred as pairs or triplets of bursts (a neurogram corresponding to this activity is shown in inset of B). Note that the unit in panel C had a characteristic peak interspike interval of ~90 ms (corresponding to 110 Hz instantaneous frequency) during ongoing activity, but that this peak interspike interval fell during the period of CRF-evoked increased mean discharge. (D) The sole unit inhibited by iv CRF. The period of clustered activity pre injection was ~70 s.

**Fig. 6 – Single-unit afferent responses to gastric distention.** Among VA units responsive during gastric distention (2 ml), two classes of response were observed. A,B: mechanoreceptive
responses temporally correlated with the distention stimulus, with peak discharge occurring
during application of peak pressure. C,D: delayed responses with peak discharge occurring
several seconds after the peak in pressure and long-lasting after-discharge, often organized into
spike pairs or bursts. Insets illustrate superimposed spike waveforms for each unit.

Fig. 7 – Comparison of efferent activity with activity of CRF-responsive single afferent
units recorded simultaneously. The two units shown are those for which the latency to the
onset of response was shortest. (A) Upper panel shows the histogram of multi-unit efferent (MU-
VED) activity inhibited by iv CRF (upper trace), and an instantaneous frequency plot of a
simultaneously recorded VA single unit excited by iv CRF (lower trace). Lower panel shows the
same plots at higher temporal resolution, demonstrating that for this VA unit, the increase in VA
activity was approximately concomitant with the decrease in VE activity. This was the only unit
for which this was the case. (B) Another VA unit recorded in the same preparation as that shown
in (A), with a similar pattern of excitation, though at much lower firing rates and a latency to
response of >5 min post injection. (C) In the sole VA unit inhibited by iv CRF, the decrease in
activity relative to the pre-stimulus ongoing pattern (instantaneous frequency plot, lower trace)
did not occur until >1 min later than the onset of iv CRF-induced inhibition of multi-unit VE
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