British Journal of Pharmacology (2002) 136, 237–247

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Intracisternal urocortin inhibits vagally stimulated gastric motility in rats: role of CRF_2

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1 Corticotropin-releasing factor (CRF) acts in the brain to inhibit thyrotropin-releasing hormone (TRH) analogue, RX-77368-induced vagal stimulation of gastric motility. We investigated CRF receptor-mediated actions of rat urocortin (rUcn) injected intracisternally (ic) on gastric motor function.

2 Urethane-anaesthetized rats with strain gauges on the gastric corpus were injected i.c. with rUcn and 20 min later, with i.c. RX-77368. CRF antagonists were injected i.c. 10 min before rUcn.

3 RX-77368 (1.5, 3, 10, 30 and 100 ng, i.c.) dose-dependently increased corpus contractions, expressed as total area under the curve (AUC, mV min^{-1}) to 2.6 ± 2.5, 6.1 ± 5.9, 9.8 ± 2.6, 69.7 ± 21.7 and 74.9 ± 28.7 respectively *vs* 0.2 ± 0.1 after i.c. saline. Ucn (1, 3 or 10 μg) inhibited RX-77368 (30 ng)-induced increase in total AUC by 28, 62 and 93% respectively *vs* i.c. saline + RX-77368.

4 The CRF_1/CRF_2 antagonist, astressin-B (60 μg, i.c.) completely blocked i.c. rUcn (3 μg, i.c.)-induced inhibition of gastric motility stimulated by RX-77368 (30 ng).

5 The selective CRF_2 antagonist, $astressin_2$-B (30, 60 or 100 μg, i.c.) dose-dependently prevented i.c. rUCn action while the CRF_1 antagonist, NBI-27914 did not.

6 In conscious rats, rUcn (0.6 or 1 μg, i.c.) inhibited gastric emptying of an ingested chow meal by 61 and 92% respectively. rUcn action was antagonized by $astressin_2$-B.

7 These data show that i.c. rUcn acts through CRF_2 receptors to inhibit central vagal gastric contractile response and postoprandial emptying.

British Journal of Pharmacology (2002) **136**, 237–247

Keywords: Astressin B; $astressin_2$-B; corticotropin-releasing factor; corticotropin-releasing factor receptor 2; gastric contractility; intracisternal; NBI-27914; RX-77368; thyrotropin-releasing hormone; urocortin; gastric emptying

Abbreviations: AUC, area under the curve; CRF, corticotropin-releasing factor; DMN, dorsal motor nucleus; DVC, dorsal vagal complex; TRH, thyrotropin-releasing hormone; rUcn, rat urocortin

Introduction

Corticototropin-releasing factor (CRF) is well established to act centrally to inhibit gastric motor function (Taché *et al*., 2001, review). CRF injected into the cerebrospinal fluid at the level of the lateral ventricle (i.c.v.), third ventricle or cisterna magna (i.c.) or microinjected into specific hypothalamic or medullary nuclei delays gastric emptying of acaloric (methycellulose) and caloric (glucose) liquids as well as gastric transit of a solid chow meal in conscious rats, and dogs (Taché *et al*., 1987; Lenz *et al*., 1988a; Sheldon *et al*., 1990; Mönnikes *et al*., 1992; 1994; Smedh *et al*., 1995; Lee & Sarna, 1997; Martínez *et al*., 1998a). In addition, CRF injected i.c. or into the dorsal vagal complex (DVC) inhibits vagally mediated high amplitude gastric contractions induced by the stable thyrotropin-releasing hormone (TRH) analogue, RX-77368 co-injected with CRF into the DVC in urethane anaesthestized fasted rats (Garrick *et al*., 1988; Heymann-Mönnikes *et al*., 1991). In conscious fasted dogs, i.c.v. injection of CRF abolished the phase III of the migrating motor complex cyclic activity front in the antrum, and induced a long-lasting disruption of interdigestive gastric cyclic contractions (Buéno & Fioramonti, 1986; Buéno *et al*., 1986). Recently, rat urocortin (rUcn) has been characterized as a 40 amino-acid peptide which shares 45% homology with rat/human (r/h)CRF. However, so far only one study investigated the central action of rUcn on gastric motor function (Kihara *et al*., 2001) and showed that the i.c.v. injection of rUcn decreased antrum motility under conditions of fed state while disrupting the fasted motor patterns of gastroduodenal activity to induce a fed-like motor pattern in conscious rats.

CRF and Ucn biological actions are mediated through binding to two high-affinity membrane-bound receptors, the subtype 1 (CRF_1) and the subtype 2 (CRF_2) which are coupled to G_s to stimulate cyclic AMP formation (Chalmers *et al*., 1996; Perrin & Vale, 1999). In rats, two CRF_2 splice variants, α and β, differing in their N-terminal domains, have been identified (Perrin & Vale, 1999). CRF_1 and CRF_2 receptors display distinct affinities for the mammalian members of the CRF family of

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peptides. In cells transfected with CRF receptors, rUcn binds with equal high affinity to both CRF receptor subtypes and their variants while r/hCRF displays high binding affinity to CRF_1 receptor, almost equal to that of rUcn, but is 40 fold less potent at the $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors (Vaughan *et al*., 1996; Perrin *et al*., 1999). Recently two new peptide members of the CRF family, urocortin II and urocortin III, have been identified by sequence homology from the human genome data base and the mouse orthologs cloned (Reyes *et al*., 2001; Lewis *et al*., 2001). Both mouse and human urocortin II and urocortin III are highly selective for the type 2 CRF receptors and exhibit low affinities for the CRF_1 receptor (Reyes *et al*., 2001; Lewis *et al*., 2001).

Studies with the specific, non-selective CRF_1/CRF_2 receptor peptide antagonists, α-helical r/h CRF_{9-41}, and the more potent [D-Phe^{12},Nle^{21,38},C$^{\gamma}$ MeLeu^{37}]r/hCRF_{12-41} and astressin (Rivier *et al*., 1984; Hernandez *et al*., 1993; Gulyas *et al*., 1995) established that central CRF-induced inhibition of gastric motor function is CRF receptor mediated in rats and dogs (Lenz *et al*., 1988b; Sheldon *et al*., 1990; Taché *et al*., 1991; Sütó *et al*., 1994; 1996; Smedh *et al*., 1995; Lee & Sarna, 1997; Martínez *et al*., 1997; 1998a; 1999; Rivier *et al*., 1998). The central action of rUcn to alter gastric motility in fed or fasted state was also prevented by the i.c.v. injection of α-helical r/hCRF_{9-41} (Kihara *et al*., 2001). However, the CRF receptor subtype involved in the central action of CRF or Ucn to inhibit gastric motor function is still to be characterized. Indirect pharmacological evidence based on the use of a selective CRF_1 receptor antagonist, and non-mammalian CRF-related peptides with higher affinity than r/hCRF to the CRF_2 receptor pointed to a role of CRF_2 receptor in mediating i.c. r/hCRF-induced delayed gastric emptying of Purina chow ingested by rats previously food deprived (Martínez *et al*., 1998a). A major breakthrough has been the development of selective CRF_2 receptor antagonists, namely, antisauvagine-30 and more recently, the long-acting derivative, astressin$_2$-B which provided new tools to assess the role of CRF_2 receptor in the biological actions of CRF and related peptides (Ruhmann *et al*., 1998; Rivier *et al*., 2001; Higelin *et al*., 2001; Million *et al*., 2002).

In the present study, we investigated the actions of rUcn injected i.c. on gastric motor functions in rats. First, we assessed the dose-related effect of i.c. rUcn on RX-77368 injected i.c. at a dose inducing a plateau stimulation of corpus contractility in urethane-anaesthetized rats. To gain insight into the CRF receptor subtype involved in rUcn inhibitory action under these conditions, we used the recently developed long acting and potent CRF_1/CRF_2 receptor antagonist, astressin B, cyclo(30–33) [D-Phe^{12},Nle^{21,38},Glu^{30}, Lys^{33},C$^{\alpha}$MeLeu^{27,40}]Ac-r/h CRF_{9-41} (Rivier *et al*., 1999), the non-peptide selective CRF_1 receptor antagonist NBI-27914 (Maciejewski-Lenoir *et al*., 2000) and the CRF_2 antagonist, astressin$_2$-B, cyclo(31-34)[D-Phe^{11},His^{12} Nle^{17},C^{d}MeLeu^{13,39},Glu^{31},Lys^{34}]Ac-Sauvagine$_{(8-40)}$ which has similar affinity to the CRF_2 variants (Rivier *et al*., 2001). Lastly, we investigated in conscious rats whether the i.c. injection of rUcn influences gastric emptying of a solid meal and the mediation of rUcn action through the CRF_2 receptor.

Methods

Animals

Male Sprague–Dawley rats (Harlan Laboratories, San Diego, CA, U.S.A.) weighing 250–300 g were housed under conditions of controlled temperature (20±3°C) and illumination (12 h light cycle beginning at 06:00). Rats were maintained with Purina Laboratory Chow (Ralston, Purina, St. Louis, MO, U.S.A.) and tap water *ad libitum*. Animals were fasted, with free access to drinking water, 18 h before the experiments. All procedures were approved by the Veterans Affairs West Los Angeles Animal Research Subcommittee (protocol no. 99-127-07).

Substances used

RX-77368 (Ferring Pharmaceuticals, Feltham, Middlesex, U.K.) was stored in a stock solution (10 ng μl^{-1} , 0.1% bovine serum albumin/saline) at −70°C. Aliquots were diluted in sterile, pyrogen-free 0.9% saline (Sigma Chemical Co, St Louis, MO, U.S.A.) immediately before use. Astressin B, astressin$_2$-B and rUcn (Clayton Foundation Laboratories for Peptide Biology, Salk Institute, La Jolla, CA, U.S.A.) were synthesized as previously described (Gulyas *et al*., 1995; Vaughan *et al*., 1996; Rivier *et al*., 2001) and kept in powder form at −70°C. Immediately before injection, astressin B and astressin$_2$-B were dissolved in double-distilled water (pH 7.0) and rUcn in saline or water. The non-peptide selective CRF_1 receptor antagonist, NBI-27914 (tosylate salt; Neurocrine Biosciences, La Jolla, CA, U.S.A.) was dissolved in 100% dimethyl sulfoxide (DMSO, Sigma) and further diluted with equal volume of saline (pH 5.8–6.0). Respective vehicles (saline, water or DMSO/saline with pH adjusted to that of NBI-27914) were used in control groups. All i.c. injections, except otherwise stated, were performed in 5 μl over 1 min followed by an additional 5 μl of saline to flush the dead space of the catheter between successive injections. All substances injected i.c. are expressed in μg per rat.

Surgery

Anaesthesia All surgical procedures and experimental protocols except otherwise stated, were performed in rats under urethane anaesthesia. Urethane (1.5 g kg^{-1}) was injected intraperitoneally (i.p.) 30 min before the start of surgery with an additional i.p. injection (0.1–0.25 g kg^{-1}) immediately before surgery if, as was usually the case, the corneal reflex was present. Adequate depth of anaesthesia was monitored at regular intervals by the absence of response to ear-pinch and corneal reflex. A temperature-controlled heating pad was used to maintain rectal temperature within 35.5–37.5°C throughout the experiment.

Cannulation of the cisterna magna The cisterna magna was cannulated as previously described (Kiraly *et al*., 1994). After the tracheotomy performed to facilitate ventilation, the head of the rat was positioned in a stereotaxic apparatus (Kopf, Model 900), and the atlanto-occipital membrane was exposed. A 25-gauge needle was inserted perpendicularly into the membrane to two-thirds of the depth of its bevel, at approximately 1.5 mm caudal to the edge of the occipital

bone, then removed. A PE-10 polyethylene catheter (Intramedic, Becton Dickinson, Parsippany, NJ, U.S.A.; length: 6 cm) was inserted 2 mm in length through the hole into the cisterna magna. The PE-10 catheter was connected to an injection port comprising a short length of stainless steel tubing joined to a PE-20 polyethylene tubing (total dead space: 5 μl). Successful cannulation was verified by leakage of clear cerebrospinal fluid from the distal catheter. If blood was observed, the experimental procedure was not further pursued. A drop of cyanoacrylate glue (Duro® SuperGlue, Rocky Hill, CT, U.S.A.) was used to hold the catheter in place, and when secured, it was covered with cotton and the incision was sutured. The cannula was capped to prevent leakage. In all experiments, we reconfirmed the correctness of the intracisternal cannula placement immediately after euthanasia. This was performed by injecting methylene blue (Sigma) under similar conditions of administration as i.c. injection of tested compounds and assessing the distribution of the dye inside the cisterna magna by visual inspection after euthanasia.

Intravenous catheter A PE-50 catheter was inserted into the right external jugular vein, and sterile, pyrogen-free 0.9% saline (Sigma) was continuously infused at a rate of 1.2 ml h^{-1} with a syringe pump to maintain hydration.

Implantation of gastric strain gauge A strain gauge (Micro-Measurement Inc; Raleigh, NC, U.S.A.; size: $10 \times 7 \times 1$ mm) was sutured to the serosa side of gastric corpus, parallel to circular smooth muscles in the greater curvature side, using Tevdek II 7-0 suture (Deknatel; Fall River, MA, U.S.A.). Then, the abdomen was closed with a suture using 3-0 silk.

Data acquisition and analysis of gastric contractility

Recording was performed in urethane-anaesthetized rats kept in supine position during the whole process. The strain gauge was connected to a physiological recorder (Gilson model 5/6H; Middleton, WI, U.S.A.). The Wheatstone bridge was balanced with 350 Ω-resistors and activated with 0.9 V DC; a high-frequency (low-pass) filter was set at 0.5 Hz. The recording was relatively free of artifacts and had minimal base-line drift, consistent with our previous report (Garrick *et al*., 1986). Strain gauge data were acquired online at a sampling rate of 6 Hz *via* a data acquisition board (AT-MIO-16E-10, National Instruments, Dallas, TX, U.S.A.) and stored in a Pentium class PC running a proprietary software program for data acquisition (LabView, National Instruments, Alfred Bayati, Astra-Zeneca, Mölndal, Sweden). Acquired strain gauge data were exported as ASCII text and imported into the digital signal processing system DADisp (DSP Development Corp., Newton, MA, U.S.A.). Strain gauge data were hi-pass filtered using a digital infinite impulse response Butterworth filter with stop frequency of 0.3 Hz, and the filtered trace was rectified. A DADisp worksheet was constructed to calculate parameters describing the intensity and duration of contractile activity. Contractile activity per minute was calculated as the area under the rectified strain gauge signal curve per minute (AUC min^{-1}) for the entire experimental period. Basal AUC was calculated as the area under the rectified strain gauge trace for the 10 min immediately preceding i.c. RX-77368 injection. The threshold for detecting an increase in corpus contractions was defined as two standard deviations above the mean AUC min^{-1} for the 10 min (basal period) before i.c. RX-77368 injection. The onset of increased AUC min^{-1} was taken as the first min of 3 consecutive minutes during which AUC min^{-1} exceeded the threshold response. The duration of increased AUC was taken as the time from onset of increased AUC min^{-1} to the first of 3 consecutive minutes during which AUC min^{-1} was below the threshold. Total AUC was calculated as the sum of AUC min^{-1} during the period between onset and termination of the response. The maximal AUC min^{-1} (peak response), the latency from i.c. RX-77368 injection to peak AUC min^{-1}, and the mean amplitude and duration of individual spikes in the rectified trace during the 5-min of maximum AUC min^{-1} were calculated.

Gastric emptying of a nutrient solid meal

The measurement of gastric emptying of a solid meal in conscious rats was performed using similar method as previously described (Martínez *et al*., 1998a; Asakawa *et al*., 1999). Fasted rats had free access to water and pre-weighed Purina chow for a 3-h period, then rats were injected i.c. with rUcn or saline and no longer had access to water or food. Gastric emptying of the ingested meal was assessed 5 h after treatment. Animals were euthanized by CO_2 inhalation followed by thoracotomy. The abdominal cavity was opened, the pylorus and cardia were clamped, and the stomach was removed. The food content in the stomach was calculated as the difference between the total weight of the stomach with its content and the weight of the stomach after the content was removed. The solid food ingested by each rat before treatment was determined by the difference between the weight of the Purina chow before feeding and the weight of the pellet chow and spill at the end of the 3-h feeding period. Gastric emptying during the 5-h experimental time was calculated according to the following equation: gastric emptying (% in 5 h) = (1 − gastric content weight/food intake weight) × 100.

Experimental protocols

Gastric motility in urethane-anaesthetized rats After the completion of surgical procedures (average 60 min), and the stabilization period (30 min), gastric corpus contractions were monitored for 30 min before and 120 min after i.c. injection of RX-77368 in urethane-anaesthetized rats fasted for 18-h.

Dose-related effects of RX-77368 and urocortin injected i.c. In the first study, after a 30 min basal period, RX-77368 (1.5, 3, 10, 30, and 100 ng) or saline was injected i.c. in separate groups of rats. Based on the dose-response curve, RX-77368 at 30 ng, which induces a plateau gastric contractile response, was selected to stimulate gastric contractility in all further experiments. In the second study, separate groups of rats were injected i.c. with rUcn at 1, 3, or 10 μg or double-distilled water and 20 min later, with RX-77368 (30 ng, i.c.).

Effects of CRF receptor antagonists on urocortin action The non-selective CRF_1/CRF_2 receptor antagonist, astressin B (30 or 60 μg), the selective CRF_1 receptor antagonist, NBI-27914 (50 or 100 μg), the selective CRF_2 receptor antagonist, $astressin_2$-B (30, 60 or 100 μg), or respective vehicles was

injected i.c. 10 min before rUcn (3 μg, i.c.) or i.c. water, and RX-77368 (30 ng) was injected i.c. 20 min later. The dose of rUcn was selected based on the above dose-response study. The dose of NBI-27914 was based on previous studies (Martínez & Taché, 2001).

Gastric emptying in conscious rats

Dose-related effect of urocortin injected i.c. Rats fasted for 18-h were given *ad libitum* access to water and Purina chow for a 3-h period, then were injected i.c. with either saline (10 μl) or rUcn (0.3, 0.6 or 1 μg in 10 μl) by puncturing the occipital membrane under short enflurane anaesthesia (2–3 min, 5% vapor concentration in O_2; Ethrane-Anaquest, Madison, WI, U.S.A.), as previously described (Martínez *et al*., 1998a). The selection of rUcn dose range was based on our previous dose response studies in conscious rats in which i.c. injection of r/hCRF or non-mammalian CRF-related peptides (0.1–1 μg) inhibited gastric emptying of non-nutrient and nutrient meals (Taché *et al*., 1987; Martínez *et al*., 1998a).

Effect of astressin$_2$-B on urocortin action Rats fasted for 18-h were given *ad libitum* access to water and Purina chow for a 3-h period, then either water (5 μl) or astressin$_2$-B (10 μg in 5 μl) followed by that of saline (5 μl, i.c.) or rUcn (1 μg in 5 μl, i.c.) were injected i.c. The dose of astressin$_2$-B was selected to provide an initial 10 : 1 antagonist : agonist ratio. In both studies, after the i.c. injections, fed rats were returned to their individual home cages without food and water, and 5-h later, were euthanized to measure gastric emptying of the meal ingested before the i.c. injection.

Statistical analysis

All results are expressed as mean ± s.e.m. Comparisons within multiple groups were performed using one-way ANOVA followed by a Student–Newman–Keuls multiple comparison test. P values less than 0.05 were considered statistically significant.

Results

Dose-related stimulation of gastric motility induced by i.c. RX-77368

In fasted urethane-anaesthetized rats, gastric contractility recorded from the strain gauge implanted onto the corpus was characterized by a uniform pattern of quiescent activity as monitored during the 30 min before and 120 min after the i.c. injection of saline. Basal AUC during 10 min immediately before the i.c. injection of RX-77368 was low (1.2 ± 0.6 mV min^{-1}), and did not differ between any pretreatment groups ($P = 0.66$). RX-77368 injected i.c. at 1.5, 3, 10, 30 and 100 ng increased dose-dependently total AUC (mV min^{-1}) to 2.6 ± 2.5 ($P > 0.05$, $n = 4$), 6.1 ± 5.9 ($P > 0.05$, $n = 4$), 9.8 ± 2.6 ($P > 0.05$, $n = 4$), 69.7 ± 21.7 ($P < 0.05$, $n = 4$) and 74.9 ± 28.7 ($P < 0.05$, $n = 3$) respectively compared with i.c. injection of saline (0.2 ± 0.1 mV min^{-1}, $n = 4$) (Figure 1). The duration of elevated AUC induced by i.c. RX-77368 was dose-related and lasted 1.3 ± 0.9, 6.5 ± 5.5, 37.5 ± 27.6, 52.8 ± 13.5 and 70.3 ± 11.6 min at 1.5, 3, 10, 30 and 100 ng respectively. The gastric contractile response to i.c. RX-77368 reached a plateau at 30 and 100 ng as shown by the total AUC (Figure 1). The minimal dose of RX-77368 (30 ng, i.c.) inducing a maximal response under our experimental conditions was selected in all subsequent studies to stimulate gastric corpus contractility.

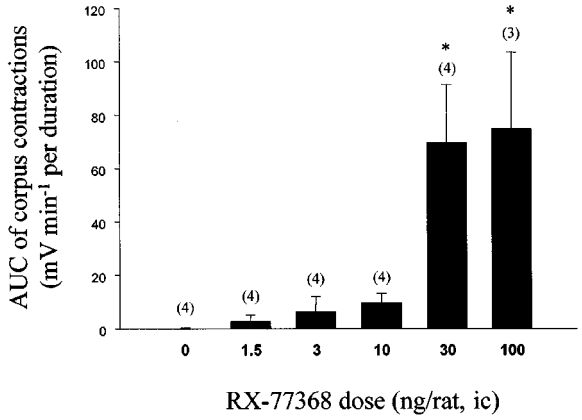

Figure 1 Dose-related stimulation of gastric contractions induced by intracisternal injection of the TRH analogue, RX-77368 in 18-h fasted urethane-anaesthetized rats. After a 30 min basal period, different groups of rats were injected i.c. with either saline or RX-77368 (1.5, 3, 10, 30 and 100 ng) and corpus contractions were recorded for 120 min. Each bar represents the mean ± s.e.m. of total AUC of gastric contractions; the number of rats per group is indicated on top of bars; *$P < 0.05$ compared with other doses (ANOVA).

Inhibitory effects of i.c. rUcn on gastric contractility stimulated by i.c. RX-77368 in urethane-anaesthetized rats

In urethane-anaesthetized rats pretreated with an i.c. injection of vehicle (water), RX-77368 (30 ng, i.c.) induced a significant stimulation of corpus contractility as assessed by the time course of changes in corpus contractile activity (Figure 2a,c) compared with the control group injected i.c. twice with vehicle (Figure 2b). Gastric contractile response, as monitored by the AUC min^{-1}, was initiated within 4.5 ± 1.6 min after the i.c. injection of RX-77368 (30 ng), peaked at 14.2 ± 3.6 min, then the response was sustained with a gradual return to pre-injection levels within 65.7 ± 9.7 min post injection (Figure 2a). Urocortin alone (10 μg, i.c.) did not influence the low basal gastric contractility compared to vehicle control (0.3 ± 0.3 *vs* 0.5 ± 0.5 mV min^{-1}, $P > 0.05$) in urethane-anaesthetized rats as shown in representative traces (Figure 2a,f), total AUC, mean spike amplitude and spike duration (Figure 3a–c). However, rUcn pretreatment (1, 3 or 10 μg, i.c.) dose dependently reduced the gastric contractile response to i.c. RX-77368 (30 ng) compared with vehicle pretreated rats as illustrated in representative traces (Figure 2a,d–f) and calculated parameters of gastric motility (Figure 3). The total AUC (mV min^{-1}) which was 63.5 ± 6.3 in i.c. vehicle + RX-77368 group, was reduced to 46.0 ± 4.7 ($P < 0.05$), 24.4 ± 4.6 ($P < 0.05$) and 4.6 ± 1.2 ($P < 0.05$) by i.c. rUCn at 1, 3 and 10 μg respectively (Figure 3a). This was associated with a reduction in contractile mean spike amplitude (Figure 3b), and mean spike duration (Figure

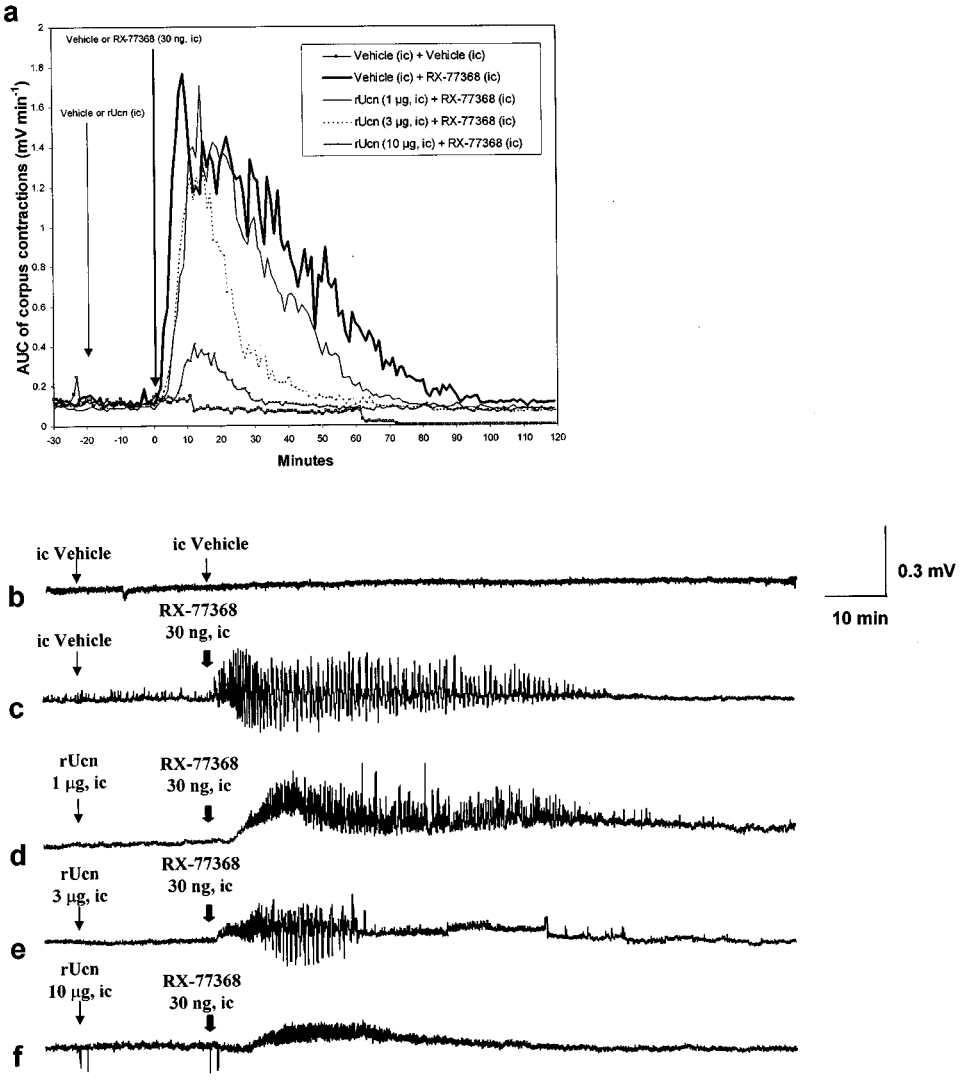

Figure 2 (a) Time course of gastric contractile response to i.c. RX-77368 and dose-related inhibitory effect of i.c. urocortin in 18-h fasted urethane-anaesthetized rats. Each point represents the mean AUC min^{-1} of corpus contractions; (b–f) Representative traces of the dose-related and temporal inhibitory effect of intracisternal urocortin on RX-77368-induced stimulation of gastric contractions. Note the marked increase in corpus contractility induced by i.c. injection of the TRH analogue (c) compared with that of saline (b) and the dose-related inhibition of contractile response (magnitude and duration) by urocortin at 1, 3 and 10 μg (d–f).

3c), most prominently at 10 μg. The duration of RX-77368 action was also dose-dependently reduced by i.c. rUcn at 3 and 10 μg to 39.1 ± 7.5 ($P<0.01$), and 14.8 ± 2.7 min ($P<0.01$) respectively compared with 65.7 ± 9.7 min in i.c. vehicle plus RX-77368. The rUcn dose of 3 μg was selected in subsequent experiments to assess the CRF receptor subtype involved in rUcn inhibitory action.

Effects of CRF receptor antagonists on i.c. rUcn-induced inhibition of RX-77368-stimulated gastric contractility in urethane-anaesthetized rats

Astressin B injected i.c. at 60 μg completely antagonized the inhibitory effects of i.c. rUcn (3 μg) on i.c. RX-77368-induced stimulation of gastric motility while at a lower dose (30 μg), astressin B had no effect (Figure 4). Likewise, the CRF_2 receptor antagonist, $astressin_2$-B (30, 60 and 100 μg) dose-dependently blocked the inhibitory effect of rUcn on corpus contractility stimulated by i.c. RX-77368 (Figure 5a). The highest dose resulted in a complete antagonism of i.c. rUcn action throughout the experimental period as shown on the time course representation of AUC (Figure 5b). By contrast, the selective CRF_1 receptor antagonist NBI-27914 (50 or 100 μg, i.c.) did not influence i.c. rUcn-induced inhibition of stimulated gastric contractility (Table 1). Neither astressin B (60 μg) nor $astressin_2$-B (100 μg) alone influenced significantly the gastric contractile response to RX-77368 (Figures 4,5a) while NBI-27914 induces a significant 40% reduction (Table 1). Representative traces of corpus contractile response to pretreatment with i.c. vehicle, astressin B (30 or 60 μg), $astressin_2$-B (100 μg) and NBI-27914 (100 μg) on i.c. rUcn-induced inhibition of corpus contractility stimulated by i.c. RX-77368 are illustrated in Figure 6b–f respectively.

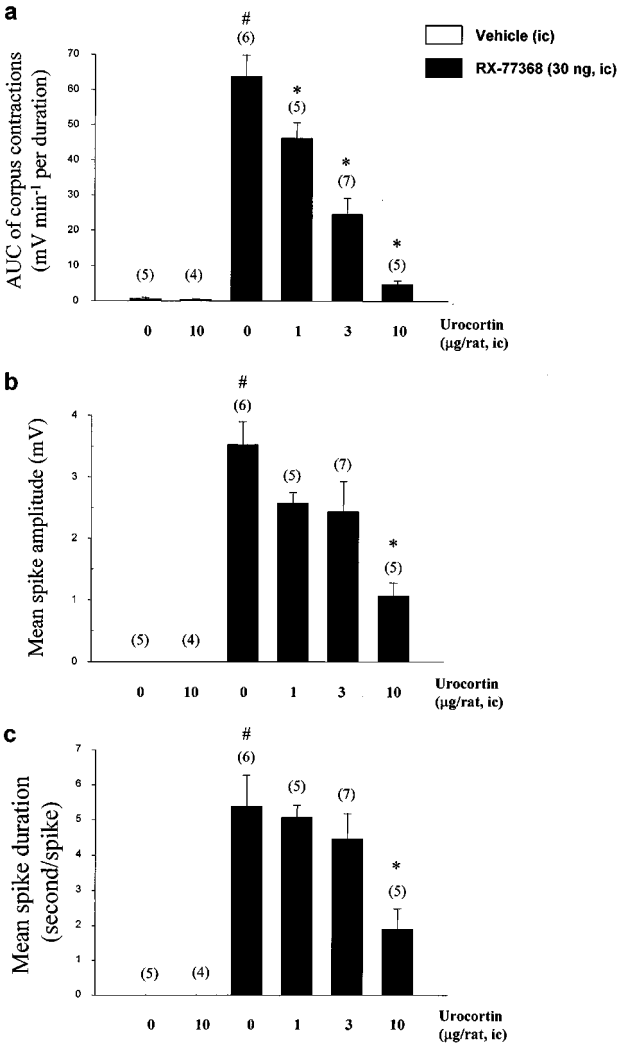

Figure 3 Dose-related inhibitory effect of intracisternal urocortin on RX-77368-induced stimulation of gastric contractions in 18-h fasted urethane-anaesthetized rats. After 30 min basal period, different groups of rats were injected i.c. with vehicle or rUcn (1, 3 or 10 μg) and 20 min later with RX-77368 (30 ng) or saline, and corpus contractions were recorded for 120 min. Bar graphs represent the mean $\pm$ s.e.m. of total AUC (a), mean spike amplitude (b), and mean spike duration (c); the number of animals is indicated on top of the columns. $^*P<0.05$ compared with vehicle + RX-77368; $\#P<0.05$ vs vehicle + vehicle or urocortin + vehicle.

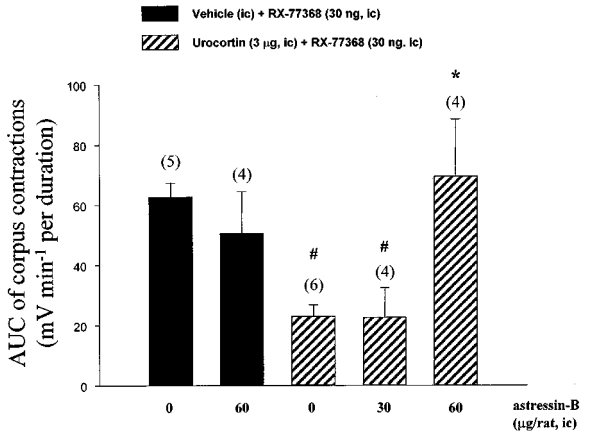

Figure 4 Intracisternal astressin B antagonized intracisternal urocortin-induced inhibition of RX-77368 induced stimulation of gastric contractions in 18-h fasted urethane-anaesthetized rats. Intracisternal injection of astressin B or vehicle was followed 10 min later by that of urocortin or vehicle and 20 min later by RX-77368. Gastric contractility was monitored for 40 min before and 120 min after RX-77368 injection. Each bar represents the mean $\pm$ s.e.m. of total AUC of corpus contractions; the number of rats per group is indicated on top of each bar. $^*P<0.05$ compared with vehicle + urocortin + RX-77368; $\#P<0.05$ compared with vehicle + vehicle + RX-77368.

Effects of i.c. rUcn and i.c. astressin$_2$-B on gastric emptying of a solid meal in conscious rats

Groups of rats fasted for 18 h, ate similar amounts of Purina chow (6.0 $\pm$ 0.9, 5.9 $\pm$ 0.8, 5.8 $\pm$ 0.5, 6.0 $\pm$ 0.4 and 6.3 $\pm$ 0.7 g, $P>0.05$) over a 3-h feeding period before receiving either no treatment, i.c. saline or rUcn at 0.3, 0.6 or 1 μg respectively. Urocortin (0.6 and 1 μg, i.c.) inhibited dose-dependently the gastric emptying of the solid meal ingested before treatment (19.5 $\pm$ 6.6% and 4.5 $\pm$ 4.5%, respectively $P<0.05$) (Figure 7a). At the lowest dose (0.3 μg), rUcn had no effect (56.1 $\pm$ 6.5%) compared with 50.4 $\pm$ 3.2% in i.c. saline-treated group (Figure 7a) and non-treated group (58.1 $\pm$ 6.8%, $n=4$).

In animals injected with vehicle + vehicle, 54.0 $\pm$ 5.2% of the meal had emptied from the stomach 5 h after the end of the feeding period, while in vehicle + rUcn (1 μg), gastric emptying was reduced to 11.2 $\pm$ 7.0% ($P<0.05$ *vs* vehicle + vehicle; Figure 7b). Pretreatment with astressin$_2$-B (10 μg) antagonized the inhibitory effect of i.c. rUcn on gastric emptying of solid meal and values were not significantly different from those of rats injected with vehicles (Figure 7b). Astressin$_2$-B (10 μg) followed by the injection of vehicle had no effect on basal gastric emptying of a solid meal (53.3 $\pm$ 5.2%).

Discussion

In the present study, the stable TRH analogue, RX-77368 injected i.c. (1.5, 3, 10, 30 and 100 ng) dose-dependently stimulated the low basal gastric contractile activity in 18-h fasted rats anaesthetized with urethane. This was assessed by monitoring mechanical activity of the gastric smooth muscles recorded from signals generated by a strain gauge acutely implanted onto the corpus serosa. The increase of gastric motility induced by i.c. RX-77368 plateaued at 30 ng. Time course response showed a rapid onset (within 5 min) with a peak reached within 10–15 min, followed by a return to the low basal activity between 60–70 min after i.c. RX-77368 injection. Basal AUC values increased by 17 fold at the peak response to i.c. RX-77368 at 30 ng while saline had no effect. The characteristics of the gastric contractile changes analysed using computer software revealed that i.c. injection of RX-77368 increase dose-dependently the mean amplitude and mean duration of individual contractile spike and the duration of the total contractile response. Likewise, other reports indicate that i.c. injection of RX-77368 (10 or 100 ng) increased the duration of high amplitude gastric contractions as monitored using a strain gauge chronically implanted on the corpus in conscious fasted rats (Garrick *et al*., 1987) or intraluminal gastric pressure in urethane-anaesthetized rats (Raybould *et al*., 1990).

Convergent neuroanatomical, electrophysiological and functional studies established that the gastric contractile response to i.c. injection of TRH or RX-77368 is mediated by central vagal myenteric–cholinergic activation of the smooth muscles (O-Lee *et al*., 1997; Sivarao *et al*., 1997; Miampamba *et al*., 2001). TRH directly activates post-synaptic preganglionic vagal motor neurons (Travagli *et al*., 1992). We previously found that RX-77368 injected i.c. induced a sustained increase in gastric vagal efferent discharges in urethane-anaesthetized rats with a rapid onset (~3 min) and long duration (~60 min) (O-Lee *et al*., 1997) similar to the time course of increased corpus contractility observed in the present study. This supports the view that gastric changes are associated with an increased vagal efferent outflow. In addition, we recently reported that RX-77368 injected i.c. at 30 and 50 ng resulted in a plateau stimulation of gastric myenteric neuronal activity in conscious rats as shown by Fos expression in corpus and antrum myenteric neurons (Miampamba *et al*., 2001). The activation of corpus myenteric ganglia encompasses cholinergic neurons and is prevented by ganglionic blockade (Miampamba *et al*., 2001). Lastly, the stimulation of gastric contractility and emptying induced by i.c. RX-77368 or TRH is blocked by vagotomy and atropine (Garrick *et al*., 1987; Martínez *et al*., 1998b).

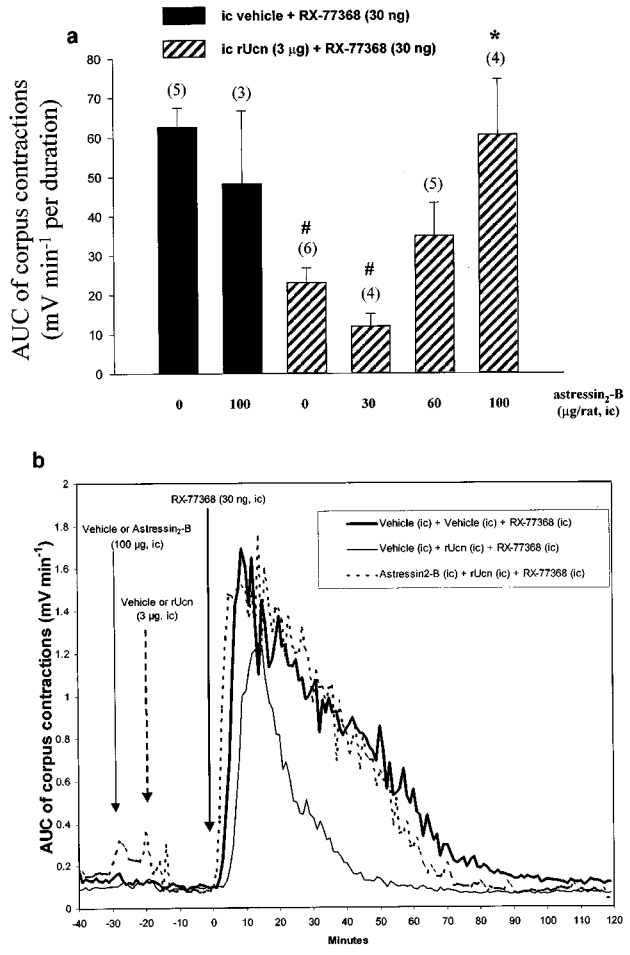

Figure 5 Intracisternal astressin$_2$-B dose-dependently antagonized intracisternal urocortin-induced inhibition of RX-77368 stimulated gastric contractions in 18 h-fasted urethane-anaesthetized rats. Intracisternal injection of astressin$_2$-B or vehicle was followed 10 min later by that of urocortin or vehicle and 20 min later RX-77368. Gastric contractility was monitored for 40 min before injections and up to 120 min after RX-77368. Each bar represents the mean ± s.e.m. of total AUC of contractions; the number of rats per group is indicated on top of each bar (a). Time course of contractile response with each point representing the mean of AUC min^{-1} (b). $*P<0.05$ compared with vehicle + urocortin + RX-77368; $\#P<0.05$ compared with vehicle + vehicle + RX-77368.

In the present study, intracisternal injection of rUcn (1, 3 and 10 μg) inhibited dose-dependently i.c. RX-77368-induced maximal stimulation of gastric corpus contractility in urethane-anaesthetized rats as assessed by the reduction in the total AUC, the mean spike amplitude and mean spike duration. Intracisternal injection of rUcn (0.6 and 1 μg) also inhibited gastric propulsive motor function in conscious rats, as shown by the dose-related suppression of postprandial gastric emptying. At 1 μg, only 9% of the food had emptied from the stomach after 5 h, indicative of the potency and long-lasting effect of i.c. rUcn inhibitory action. The observed decreased gastric emptying of the ingested meal is not confounded by a possible reduction of food ingestion reported to occur after central injection of rUcn at similar doses (Spina *et al*., 1996). In our studies, rUcn was injected i.c. after the 3-h refeeding period and the amount of Purina chow eaten by rats in all the experimental groups was similar before i.c. injection of rUcn. Moreover, animals had no access to food or water after the i.c. treatment. r/hCRF injected i.c. inhibits gastric acid secretion in conscious fasted rats (Taché *et al*., 1983). Such a possible decrease in gastric fluid secretion would reduce the weight content of the stomach, while we found an increase after i.c. rUcn, suggesting that the gastric emptying measurements reflect a delayed gastric emptying. Consistent with these observations, the non-mammalian CRF-related 40 amino-acid peptide, sauvagine which has high affinity to CRF_2 receptors, injected i.c. also completely suppressed gastric emptying of a solid meal at similar dose range (Martínez *et al*., 1998a).

Table 1 Effect of the selective CRF_1 receptor antagonist, NBI-27914 injected intracisternally on intracisternal urocortin-induced inhibition of gastric corpus contractility stimulated by RX-77368 in urethane-anaesthetized rats

Treatment	*N*	*Total AUC* (mV min^{-1})
Vehicle/vehicle/RX-77368	3	87.2 ± 19.3
NBI-27914 (100 μg)/vehicle/RX-77368	4	52.0 ± 11.3 # ($P=0.043$)
Vehicle/rUcn/RX-77368	3	25.5 ± 8.5 # ($P=0.008$)
NBI-27914 (50 μg)/rUcn/RX-77368	3	10.1 ± 3.8 # ($P=0.004$)
NBI-27914 (100 μg)/rUcn/RX-77368	4	11. ± 7.6 # ($P=0.002$)

Intracisternal (i.c.) injection of vehicle or NBI-27914 was followed 10 min later by that of i.c. rUcn (3 μg) or vehicle, and 20 min later by i.c. RX-77368 (30 ng) in urethane-anaesthetized 18 h-fasted rats. Each value represents of mean ± s.e.m. of total AUC of corpus contractions; the number of rats per group is listed in N. # compared with vehicle + vehicle + RX-77368.

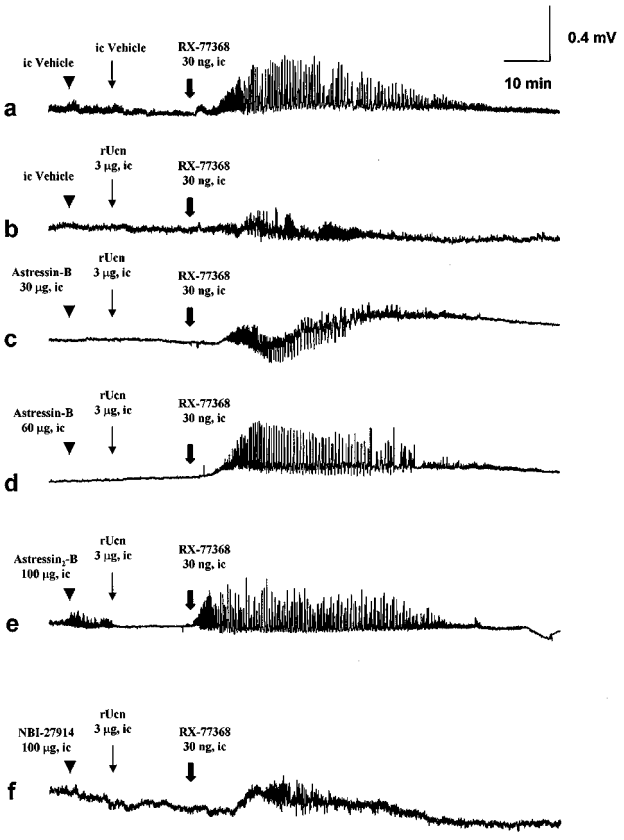

Figure 6 Representative tracing of blockade of intracisternal urocortin-induced inhibition of gastric motor activity stimulated by RX-77368: blockade by the CRF_1/CRF_2 receptor antagonist, astressin B and the selective CRF_2 receptor antagonist, astressin$_2$-B but not by the selective CRF_1 receptor antagonist, NBI-27914 in 18-h fasted urethane-anaesthetized rats. Note the marked increase in corpus contractility induced by i.c. injection of the TRH analogue (a) and the inhibitory effect of i.c. rUcn (b) and the dose-related reversal by astressin B (c,d) and astressin$_2$-B (e) while NBI-27914 had no effect (f).

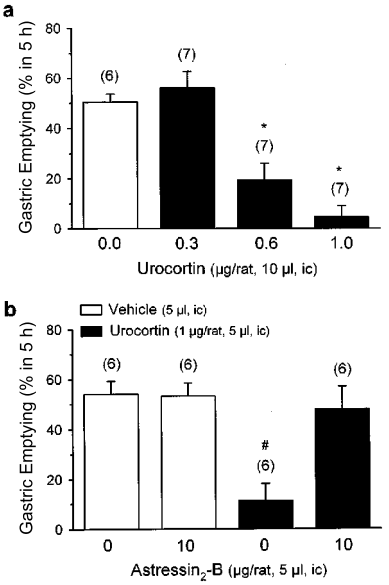

Figure 7 Dose-related inhibition of gastric emptying of a solid meal by intracisternal injection of urocortin in conscious fed rats (a) and blockade of urocortin effects by pretreatment with the selective CRF_2 receptor antagonist astressin$_2$-B (b). Fasted rats were given free access to Purina chow and water for 3 h, then food and water were removed; (a) saline or rUcn (0.3–1 μg) was injected i.c. in different groups of rats; (b) vehicle or astressin$_2$-B was injected i.c. followed by i.c. injection of saline or rUcn (1 μg) and gastric emptying monitored 5 h later. Each bar represents the mean $\pm$ s.e.m.; the number of rats per group is indicated on top of each bar. $^*P<0.05$ compared with vehicle (0.0) and rUen (0.3). $\#P<0.05$ compared with other experimental groups.

The i.c.v. injection of rUcn at 0.1–5 μg was also reported to suppress dose-dependently the antrum motor activity induced by refeeding after a fast in conscious rats (Kihara *et al*., 2001). In the present study, rUcn injected i.c. at 0.3–1 μg suppressed postprandial gastric emptying by 61–91%, while doses ranging from 1–10 μg are required to inhibit centrally stimulated gastric contractility by 29–95%. Such a difference in rUcn dose range to suppress gastric motor function may be related to the different experimental models used: conscious preparation with no surgery in gastric emptying studies *vs* urethane-anaesthetized rats with acute surgery in the gastric motility experiments. However, irrespective of animal preparations, rUcn injected into the cerebrospinal fluid inhibits gastric motor function as shown by the dose-related suppression of corpus contractions induced by central vagal stimulation in anaesthetized fasted rats (present study), decreases antral motility index associated with refeeding in conscious rats (Kihara *et al*., 2001), and slows postprandial gastric emptying in conscious rats (present study).

Additional studies using CRF receptor antagonists provided convergent evidence that i.c. rUcn inhibitory action is CRF receptor mediated and that the receptor subtype 2 is primarily involved. First, astressin B injected i.c. completely prevented i.c. rUcn-induced decrease of corpus contractility induced by i.c. RX-77368. Astressin B is a recently developed long-acting antagonist at both CRF_1 and CRF_2 receptors (Rivier *et al*., 1999). Likewise, i.c.v. rUcn-induced antral motility alteration of the fed and fasted patterns was blocked by the non-selective CRF receptor antagonist, α-helical CRF_{9-41} in conscious rats (Kihara *et al*., 2001). Second, in the present study, the selective CRF_2 antagonist, astressin$_2$-B (Rivier *et al*., 2001) injected i.c. dose-dependently abolished i.c. rUcn inhibitory action on vagally stimulated gastric motility in urethane-anaesthetized rats. The choice of astressin$_2$-B was based on the stability and selectivity of this new CRF_2-receptor antagonist in *in vitro* binding studies on transfected CRF receptors and *in vivo* studies (Rivier *et al*., 2001; Martínez *et al*., unpublished observations). Moreover, we showed that i.c. rUcn-induced inhibition of gastric emptying of a solid meal was completely antagonized by astressin$_2$-B in conscious rats. The higher antagonist : agonist ratio (33 : 1) at which i.c. astressin$_2$-B completely blocked i.c. rUcn inhibitory action on gastric motility compared with gastric emptying (10 : 1) may be related to difference in experimental conditions (anaesthetized *vs* conscious, as well as the nature of the stimuli to be inhibited by rUcn: exogenously induced maximal central vagal stimulation *vs* physiological postprandial vagal tone). By contrast, the selective CRF_1 receptor antagonist, NBI-27914 (Chen *et al*., 1996; Maciejewski-Lenoir *et al*., 2000) did not influence rUcn inhibitory action on stimulated gastric motility. Consistent

with these findings, NBI-27914 administered i.c. did not influence i.c. CRF, sauvagine and urotensin I (0.3 or 1 μg)-induced delayed gastric emptying of a solid meal in conscious rats (Martínez *et al*., 1998a). However, the CRF_1 receptor antagonist NBI-27914, injected i.c.v. at similar doses was biologically active to block i.c.v. CRF-induced colonic motor activation (Martínez & Taché, 2001). The inhibitory effect of NBI-27914 *per se* on total AUC may indicate that the compound acts as an inverse agonist under these conditions. Collectively, these observations provide strong support for the involvement of CRF_2 receptor in mediating i.c. rUcn-induced inhibition of gastric motor function.

Convergent evidence indicates that neuronal pathways, through which i.c. rUcn inhibits i.c. RX-77368 effect *via* CRF_2 receptor interaction, may take place within the DVC, involving modulation of vagal outflow to the stomach. Direct co-microinjection of CRF and RX-77368 into the DVC inhibited the stimulated gastric motility in urethane-anaesthetized rats (Heymann-Mönnikes *et al*., 1991), indicative of an interaction between CRF and the stable TRH analogue within medullary nuclei regulating gastric vagal outflow (Berthoud *et al*., 1991). Cell groups expressing the CRF_2 receptor have been localized in the area postrema and medial nucleus tractus solitarius (NTS) (Bittencourt & Sawchenko, 2000; Van Pett *et al*., 2000). Urocortin injected i.c.v. at 1 μg activates neurons in the area postrema and NTS as shown by Fos expression (Bittencourt & Sawchenko, 2000). The activation of these medullary nuclei has been associated with inhibitory influence on preganglionic vagal neurons as established by electrophysiological and functional studies (Olson *et al*., 1993; Rogers & McCann, 1993). Moreover, we reported that i.c.v. injection of CRF induced Fos expression in NTS neurons while inhibiting brain medullary TRH circuitry-induced Fos expression in the dorsal motor nucleus of the vagus (DMN) (Wang *et al*., 1996). Lastly, electrophysiological studies showed that i.c. injection of sauvagine, which has higher affinity for CRF_2 receptors than r/hCRF (Perrin & Vale, 1999), is more potent than i.c. CRF to inhibit gastric vagal efferent discharges in urethane-anaesthetized rats (Kosoyan *et al*., 1999). Functional reports in conscious rats also established that truncal vagotomy abolished the changes in antrum motility index induced by rUcn injected i.c.v. (Kihara *et al*., 2001) and the delayed gastric emptying induced by i.c. CRF or sauvagine (Taché *et al*., 1987; Broccardo & Improta, 1990).

In summary, the present studies demonstrate that i.c. injection of RX-77368 induced a dose-related stimulation of gastric corpus contractility with a plateau response observed at 30 and 100 ng in urethane-anaesthetized rats. The i.c. injection of rUcn dose-dependently inhibited corpus contractility induced by i.c. RX-77368 at 30 ng in urethane-anaesthetized rats and gastric emptying of a solid meal in conscious rats. The inhibitory action of i.c. rUcn on gastric motor function is mediated through interaction with CRF_2 receptors. This is supported by the blockade of i.c. rUcn-induced suppression of RX-77368-stimulated gastric contractility by i.c. pretreatment with the CRF_1/CRF_2 receptor antagonist, astressin B and the selective CRF_2 receptor antagonist, astressin$_2$-B while the selective CRF_1 receptor antagonist, NBI-27914 had no effect. Likewise, the selective CRF_2 receptor antagonist blocked i.c. rUcn-induced inhibition of gastric emptying. These data along with previous pharmacological evidence that non-selective CRF receptor antagonists injected centrally prevented the delayed gastric emptying induced by various stressors (Taché *et al*., 2001, review), suggest that selective CRF_2 receptor antagonist acting in the brain medulla may have relevance to alleviate stress-related inhibition of gastric motor function.

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases, R01 Grants DK-30110, DK 33061 and DK 26741 and Center grant DK-41301 (Animal Core). Chih-Yen Chen, is supported by Taiwan NHRI Physician Scientist Fellowship. The authors thank Dr Dimitri Grigoriadis (Neurocrine Bioscience, La Jolla, CA, U.S.A.) for the generous supply of NBI-27914. Dr Alfred Bayati (AstraZeneca, Mölndal, Sweden) is acknowledged for provision of a new version of Data Acquisition® software program, and Mr Paul Kirsch for his assistance in the preparation of the manuscript.

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(Received February 7, 2002
Accepted March 5, 2002)