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Sensitizing effects of lafutidine on CGRP-containing afferent nerves in the rat stomach

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1 Capsaicin sensitive afferent nerves play an important role in gastric mucosal defensive mechanisms. Capsaicin stimulates afferent nerves and enhances the release of calcitonin gene-related peptide (CGRP), which seems to be the predominant neurotransmitter of spinal afferents in the rat stomach, exerting many pharmacological effects by a direct mechanism or indirectly through second messengers such as nitric oxide (NO).

2 Lafutidine is a new type of anti-ulcer drug, possessing both an antisecretory effect, exerted *via* histamine H_2 receptor blockade, and gastroprotective activities. Studies with certain antagonists or chemical deafferentation techniques suggest the gastroprotective actions of lafutidine to be mediated by capsaicin sensitive afferent nerves, but this is an assumption based on indirect techniques. In order to explain the direct relation of lafutidine to afferent nerves, we conducted the following studies.

3 We determined CGRP and NO release from rat stomach and specific [^{3}H]-resiniferatoxin (RTX) binding to gastric vanilloid receptor subtype 1 (VR1), which binds capsaicin, using EIA, a microdialysis system and a radioreceptor assay, respectively.

4 Lafutidine enhanced both CGRP and NO release from the rat stomach induced by a submaximal dose of capsaicin, but had no effect on specific [^{3}H]-RTX and capsaicin binding to VR1.

5 In conclusion, our findings demonstrate that lafutidine modulates the activity of capsaicin sensitive afferent nerves in the rat stomach, which may be a key mechanism involved in its gastroprotective action.

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Keywords: lafutidine; CGRP; nitric oxide; VR1; afferent nerve; sensitization

Abbreviations: CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; GMBF, gastric mucosal blood flow; NO, nitric oxide; NOS, nitric oxide synthase; RTX, resiniferatoxin; VR1, vanilloid receptor subtype 1

Introduction

Recent studies have shown that capsaicin sensitive afferent nerves play an important role in gastric mucosal defensive mechanisms (Holzer, 1998). When the gastric mucosal barrier is focally disrupted and gastric acid back-diffusion occurs, chemosensitive afferent neurons function as a neuronal emergency system enhancing gastric mucosal blood flow (GMBF) and gastric mucus secretion, and facilitating gastric epithelial restitution (Holzer, 1998). Capsaicin, the pungent ingredient in red peppers and chillies, has been a pharmacological tool for the study of afferent nerves. Chronic treatment with a neurotoxic dose of capsaicin was used to achieve chronic ablation of afferent nerves (Buck & Burks, 1986), but a low dose of capsaicin showed gastroprotective actions. Intragastric administration of low dose capsaicin increased the basal gastric mucosal blood flow and gastric mucus secretion, and it also facilitated gastric epithelial restitution (Holzer *et al*., 1991; Matsumoto *et al*., 1991; Onodera *et al*., 1999a). *Via* these mechanisms, capsaicin inhibited acute gastric mucosal lesions caused by several injurious factors such as hydrochloric acid, ammonia, ethanol, aspirin and indomethacin (Onodera *et al*., 1995; Abdel-Salam *et al*., 1999). These effects of capsaicin are supressed or abolished by capsaicin induced defunctionalization of afferent nerves, tetrodotoxin treatment, the $CGRP_1$ receptor antagonist $CGRP_{8-37}$ or NO synthase inhibitor treatment (Holzer *et al*., 1991; Lambrecht *et al*., 1993; Onodera *et al*., 1999a; Whittle *et al*., 1992). CGRP, substance P and neurokinin A are bioactive peptides contained in sensory afferent nerves. One of them, CGRP, seems to be the predominant neurotransmitter of spinal afferents in the rat stomach exerting many pharmacological effects, such as vasodilating and gastroprotective actions (Evangelista *et al*., 1992; Green & Dockray, 1988; Holzer *et al*., 1990; Li *et al*., 1991), *via* a direct mechanism or indirectly through second messengers such as NO (Chen & Guth, 1995; Holzer *et al*., 1993; Lambrecht *et al*., 1993). Indeed, these nerves are found in the myenteric plexus, circular muscle and around the submucosal microvasculature (Green & Dockray, 1988; Sternini *et al*., 1987) and CGRP immunostaining is more marked in the ulcer region than in normal portions of the stomach (Tani *et al*., 1999), and the rate of repair of chronic gastric ulcers induced by acetic acid was delayed after sensory nerve ablation (Tramontana *et al*., 1994). These findings indicate that capsaicin sensitive afferent nerves exert gastroprotective effects through CGRP and in part *via* NO release,

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as well as playing an important role in the healing process of chronic ulcers.

Lafutidine is a new type of anti-ulcer drug possessing an antisecretory effect exerted *via* histamine H_2 receptor blockade (Shibata *et al*., 1993) as well as gastroprotective activity against several necrotizing agents independent of its antisecretory action (Onodera *et al*., 1995; 1999a). It also reportedly increases the gastric mucosal blood flow (Onodera *et al*., 1999a) and gastric mucus secretion (Ichikawa *et al*., 1994; 1998), and accelerates epithelial restitution in rats (Onodera *et al*., 1999a). Like the effects of capsaicin, the gastroprotective action of lafutidine was depressed or abolished by treatment with tetrodotoxin or $CGRP_{8-37}$, or chemical defunctionalization of afferent nerves (Onodera *et al*., 1995; 1999a), indicating that capsaicin sensitive nerves contribute significantly to the mechanisms underlying the actions of lafutidine. Additionally, lafutidine accelerated repair of chronic gastric or duodenal ulcer in rats and the recurrence rate of chronic gastric ulcer, induced by acetic acid or treatment of the serosa-searing with a hot metal bar, was low (Ajioka *et al*., 2000; Onodera *et al*., 1998; 1999b). These observations suggest that the effects of lafutidine are due mostly to the activation of capsaicin sensitive afferent nerves. However, this is an assumption based on indirect techniques using antagonists and chemical deafferentation. Thus, in this study, we have attempted to explain the direct relation of lafutidine to afferent nerves. For this purpose, we initially determined the amounts of CGRP and NO released from the rat stomach, and next studied the VR1 binding profile in the rat gastric mucosa.

Several candidates, including anandamide (Zygmunt *et al*., 1999) and hydroperoxyeicosatetraenoic acids (Hwang *et al*., 2000), have been reported to be endogenous activators of the capsaicin receptor, but a physiological VR1 activator has not yet determined. In this study, instead of the emergent stimulation of afferent nerves at injured gastric mucosa, we used capsaicin as a tool to stimulate afferent nerves through VR1.

Methods

Measurement of neuropeptide release

Seven-week-old male Sprague-Dawley rats (Charles River Japan) weighing 185–265 g were used. The animals were fasted for 18 h before the experiments but had free access to water. Tissue isolation procedures were conducted according to the method of Inaba *et al*. (1996). Briefly, under ether anaesthesia, rat stomachs were isolated. The forestomach and antrum were dissected free and the corpus was inverted so as to be mucosal side out. These tissues weighing 643–892 mg were rinsed and allowed to stabilize for 1 h in cold oxygenated modified Krebs-Henseleit solution (in mM: NaCl 110, KCl 4.7, $CaCl_2$ 1.0, $MgCl_2$ 1.15, $NaHCO_3$ 25, glucose 5.6 and HEPES 10, pH 7.4). The tissues were incubated in test tubes containing 2 ml of modified Krebs-Henseleit solution oxygenated with 95% O_2 and 5% CO_2. Capsaicin or vehicle was added to the incubation tube. Thirty minutes later, the incubated solution was centrifuged at 3000 r.p.m. for 5 min at 4°C. The CGRP content of the supernatant was determined by using enzyme linked immunoassay kit (SPI bio) (Frobert *et al*., 1999). Lafutidine or capsazepine was added 10 min before capsaicin treatment.

Microdialysis and measurement of NO production in the rat gastric wall

Seven-week-old male Sprague-Dawley rats (Charles River Japan) weighing 190–227 g were used. The animals were fasted for 18 h before the experiments but had free access to water. Rats were anaesthetized with urethane (1.5 g kg^{-1}, s.c.) and placed on a warmed plate maintained at 37°C. The stomach was exposed by laparotomy, and a polyethylene cannula was inserted and fixed to the forestomach for drug treatment. A microdialysis probe (S-A-20-05, 5 mm in length dialysis membrane, Eicom, Japan) was inserted into the gastric corpus wall, and was perfused with Ringer's solution (in mM: NaCl 140, KCl 4, $CaCl_2$ 1.26 and $MgCl_2$ 1.15, pH 7.4) at a constant flow rate of 2 μl min^{-1}. The perfused dialysates were collected every 10 min, and according to the methods described by Yamada & Nabeshima (1997), NO metabolites were measured using an automated NO detecter HPLC system (ENO-10, Eicom). Briefly, NO_2^- and NO_3^- in the dialysate were separated by a reverse-phase separation column (NO-PAK, 4.6 × 50 mm, Eicom), and NO_3^- was reduced to NO_2^- in a reduction column (NO-RED, Eicom). NO_2^- was mixed with a Griess reagent at 35°C and absorbance of the colour of the product dye at 540 nm was measured by a flow-through spectrophotometer (NOD-10, Eicom). The mobile phase consisted of 10% methanol containing 0.15 M NaCl-NH_4Cl and 0.5 g l^{-1} of EDTA-4Na, and was delivered by a pump at a rate of 0.33 ml min^{-1}. The Griess reagent was delivered at a rate of 0.1 ml min^{-1}. The total NO metabolite level was the sum of NO_2^- and NO_3^- in Ringer's solution, and the reliability of the reduction column was examined in each experiment. NO metabolites were measured during a 60 min period after capsaicin or vehicle (0.5% CMC) treatment, and lafutidine or vehicle (distilled water) was intragastrically administered 10 min before capsaicin treatment. All drugs were administered as a volume of 0.5 ml per 100 g body weight.

Receptor binding assay

Seven-week-old male Sprague-Dawley rats (Charles River Japan) weighing 191–289 g were used. The animals were fasted for 18 h before the experiments but had free access to water. The radioreceptor assay of [^{3}H]-RTX was performed according to a previously described method (Nozawa *et al*., 2001). Briefly, rat stomach mucosa was removed and placed in ice-cold buffer (in mM: KCl 5, NaCl 5.8, $MgCl_2$ 2, $CaCl_2$ 0.75, sucrose 137, HEPES 10, pH 7.4), then disrupted with a polytron tissue homogenizer. The homogenates were centrifuged for 10 min at 2000 *g*, the pellets were discarded and the supernatants were then centrifuged at 35,000 *g* for 30 min. The resulting pellets were resuspended in the buffer and used for the binding assay. All steps of membrane preparation were performed at 4°C. The membranes (100 μg of protein), [^{3}H]-RTX and unlabelled ligands were incubated at 37°C for 60 min in 1 ml of buffer. Specific binding was calculated experimentally using the difference between counts in the absence and presence of 1 μM nonradioactive RTX. One nM [^{3}H]-RTX, which corresponds to approximately 50% receptor occupancy

was used for the competition experiments. After the binding reaction was terminated by chilling the assay mixture on ice, 100 μg of bovine α_1-acid glycoprotein were added to each tube to reduce nonspecific binding (Szallasi *et al.*, 1992). Bound and free [^{3}H]-RTX were then separated by pelleting the membranes. The radioactivity of the pellet was determined by scintillation counting. The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]-RTX binding were estimated by Rosenthal analysis (Rosenthal, 1967) of the saturation data. The ability of displacers to inhibit specific [^{3}H]-RTX binding was estimated based on IC_{50} values, which are the molar concentrations of unlabelled displacer necessary for displacing 50% of the specific binding (estimated by log probit analysis). The value for the inhibition constant, K_i, was calculated from the equation, $K_i = IC_{50}/(1 + L/K_d)$, where L equals the concentration of [^{3}H]-RTX. The Hill coefficient for saturation binding of [^{3}H]-RTX was obtained from Hill plot analysis. The binding data were analysed using the GraphPad InPlot computer program.

Drugs

Sodium nitroprusside (SIGMA, U.S.A.) and N^G-nitro-L-arginine methyl ester (L-NAME, SIGMA, U.S.A.) were dissolved in saline. Capsaicin (Wako, Japan) was dissolved in ethanol to achieve a 1 mM concentration and diluted with assay buffer for the *in vitro* study, then suspended with 0.5% methylcellulose solution for the *in vivo* study. Capsazepine (RBI, U.S.A.) was dissolved in dimethylsulphoxide to achieve a 10 mM concentration and diluted with assay buffer. Lafutidine (Sentoraru-Garasu, Japan) was dissolved in 0.1 N HCl and neutralized with 0.1 N NaOH. For the *in vivo* study, each drug was administered intragastrically or intravenously in a volume of 0.5 or 0.1 ml per 100 g body weight, respectively.

Statistics

All values are presented as means and standard errors. Statistically significant differences between the groups were determined using Student's *t*-test or Dunnett's multiple comparison test. Statistical calculations were performed with SAS system release 6.12 (SAS Institute inc.). Values of $P < 0.05$ were judged to be statistically significant.

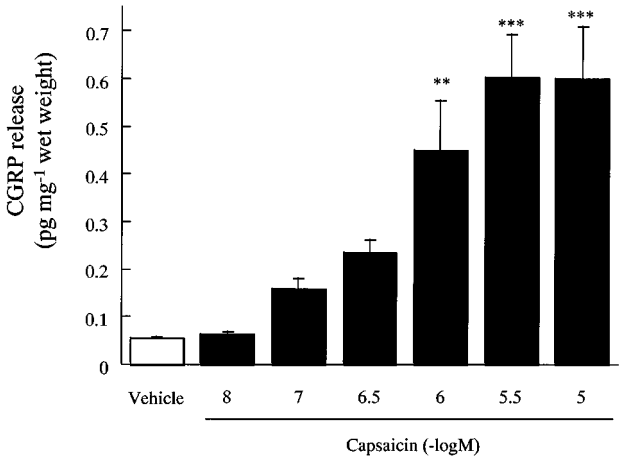

Figure 1 Effect of capsaicin on CGRP release from isolated rat stomach. The stomach was incubated with capsaicin for 30 min and CGRP in the incubation medium was determined by EIA. Each column and vertical bar represents the mean and s.e.mean of three separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$; significantly different from vehicle (Dunnett's multiple comparison test).

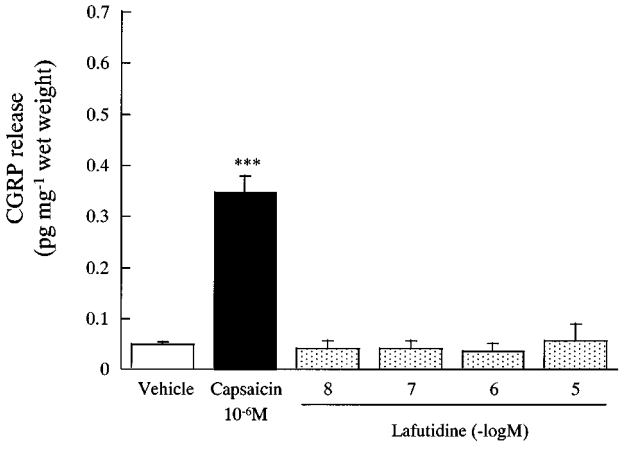

Figure 2 Effect of lafutidine on CGRP release from isolated rat stomach. The stomach was incubated with lafutidine for 30 min and CGRP in the incubation medium was determined by EIA. Capsaicin (10^{-6} M) was treated as a positive control. Each column and vertical bar represents the mean and s.e.mean of 10 separate experiments. $^{***}P < 0.001$; significantly different from vehicle (Student's *t*-test).

Results

Measurement of neuropeptide release

Capsaicin increased CGRP release from the isolated stomach in a concentration-dependent manner. The basal CGRP release was 0.054 ± 0.004 pg mg^{-1} wet weight, and capsaicin at a dose of 3×10^{-6} M induced an 11-fold increase above basal levels to a value of 0.602 ± 0.092 pg mg^{-} wet weight with 30 min of incubation (Figure 1). Lafutidine (10^{-8}–10^{-5} M) alone had no effect on basal CGRP release (Figure 2), but dose-dependently enhanced capsaicin-stimulated peptide release. At 10^{-6} M, lafutidine augmented CGRP release induced by a submaximal dose of capsaicin (3×10^{-7} M) from 0.256 ± 0.018 to 0.573 ± 0.041 pg mg^{-} wet weight (Figure 3). In addition, capsazepine (10^{-5} M) pretreatment abolished the increase in CGRP levels caused by capsaicin (3×10^{-7} M) alone or capsaicin with lafutidine (10^{-6} M), restoring the basal level (Figure 4).

Microdialysis and measurement of NO production in the rat gastric wall

Before this experiment, we confirmed that intravenous administration of sodium nitroprusside (1–3 mg kg^{-1}) and L-NAME (10 mg kg^{-1}) increased and decreased, respectively, the NO metabolite levels in the gastric wall (data not shown). In this study, intragastric administration of capsaicin (0.3–30 mg kg^{-1}) caused a dose-dependent and sustained increase in NO metabolite levels in the gastric wall (Figure 5). Intragastric administration of lafutidine (1–30 mg kg^{-1}) had no effect but lafutidine (3–30 mg kg^{-1}) pretreatment dose-dependently augmented NO production

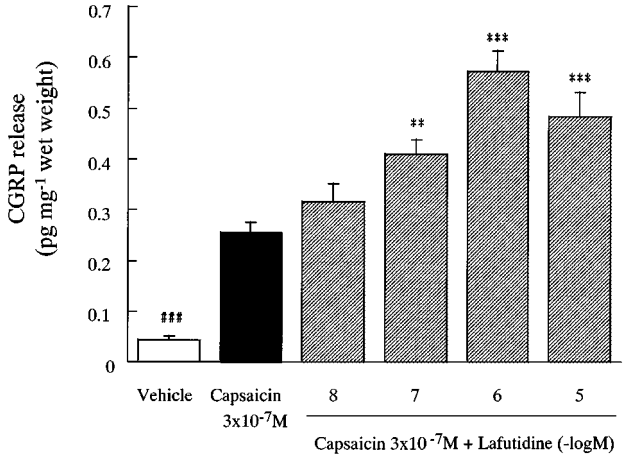

Figure 3 Effect of lafutidine on capsaicin induced CGRP release from isolated rat stomach. The stomach was incubated with capsaicin for 30 min and CGRP in the incubation medium was determined by EIA. Lafutidine was added 10 min before capsaicin treatment. Each column and vertical bar represents the mean and s.e.mean of eight separate experiments. ###$P<0.001$; significantly different from capsaicin (Student's t-test). **$P<0.01$, ***$P<0.001$; significantly different from capsaicin (Dunnett's multiple comparison test).

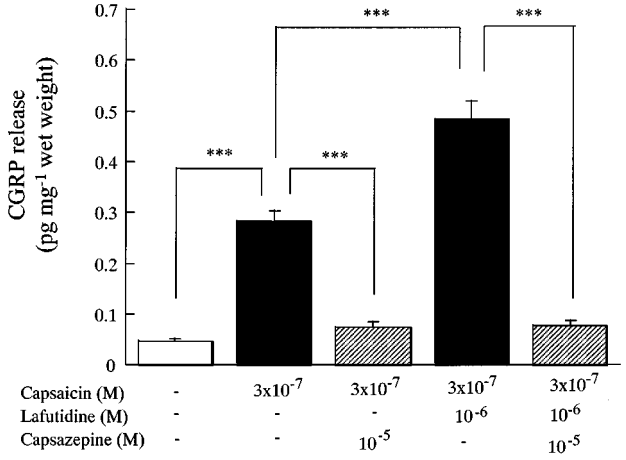

Figure 4 Effect of capsazepine on CGRP release induced by capsaicin alone or combined with lafutidine from isolated rat stomach. The stomach was incubated with capsaicin for 30 min and CGRP in the incubation medium was determined by EIA. Lafutidine or capsazepine was added 10 min before capsaicin treatment. Each column and vertical bar represents the mean and s.e.mean of eight separate experiments. ***$P<0.001$; significantly different between two groups (Student's t-test).

induced by low dose capsaicin (0.3 mg kg^{-1}) (Figure 6). The basal NO metabolite level before drug treatment in 10 μl of dialysate during a 10 min period was 199.5$\pm$7.0 pmol. We calculated changes in NO metabolite production in the 10 μl of dialysate for each 10 min period after drug treatment, and the sum of these changes for 60 min was presented as 'increased NO metabolite production'. Vehicle, lafutidine (30 mg kg^{-1}) and low dose capsaicin (0.3 mg kg^{-1}) alone showed 119.9$\pm$25.8, 157.6$\pm$18.8 and 123.0$\pm$44.9 pmol, respectively, of 'increased NO metabolite production'. In combination with capsaicin (0.3 mg kg^{-1}), lafutidine at doses of 3, 10, 30 mg kg^{-1} showed 217.0$\pm$38.6,

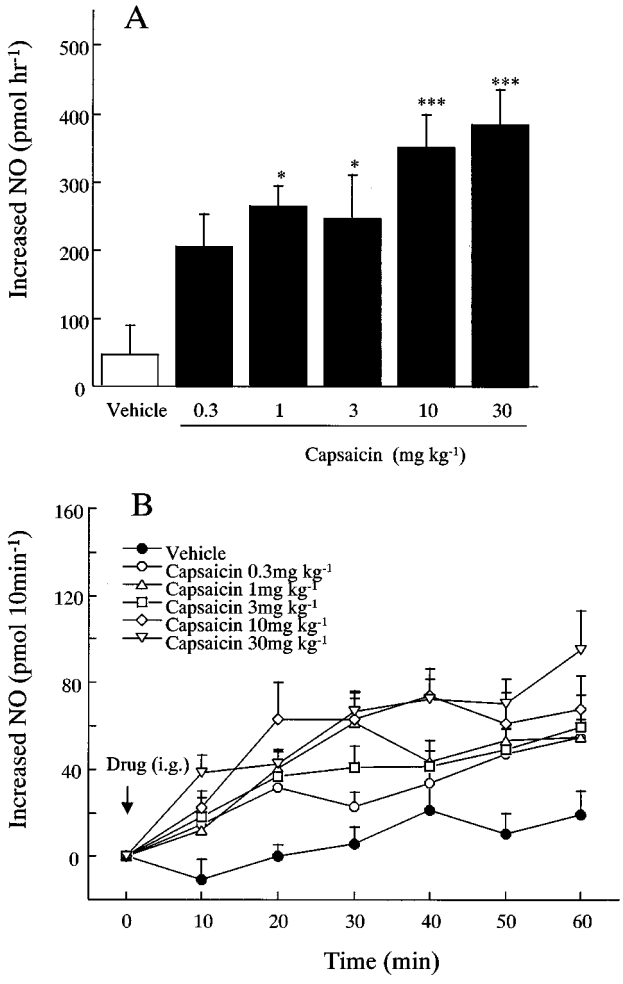

Figure 5 Effect of capsaicin on NO production from stomachs of anesthetized rats. The perfused dialysates were collected from the stomach every 10 min and NO metabolites were determined with an automated NO detector HPLC system. (A) The sum of NO production over the basal level during 60 min period were calculated. (B) Changes in NO production in the dialysate. Capsaicin was intragastrically administered through a cannula in the forestomach. Each point or column and vertical bar represents the mean and s.e.mean of five separate experiments. (A), *$P<0.05$, ***$P<0.001$; significantly different from vehicle (Dunnett's multiple comparison test).

239.1$\pm$27.8 and 411.4$\pm$90.0 pmol of 'increased NO metabolite production', respectively.

Receptor binding assay

We previously reported that [^{3}H]-RTX showed saturable specific binding to rat gastric mucosal membrane preparations (Nozawa *et al*., 2001). The effect of each drug on specific [^{3}H]-RTX binding was determined at a 1 nM [^{3}H]-RTX concentration, which corresponds to approximately 50% receptor occupancy. Unlabelled RTX and capsaicin inhibited specific [^{3}H]-RTX binding with IC_{50} values of 0.186 and 88.1 nM, respectively, but lafutidine ($10^{-9}-10^{-5}$ M) had no effect on [^{3}H]-RTX specific binding (Figure 7). Treatment with 10^{-5} M of lafutidine had no influence on [^{3}H]-RTX saturable binding. The K_d and B_{max} values for [^{3}H]-RTX binding were 1.7$\pm$0.1 nM and 135$\pm$8 fmol mg^{-1} protein

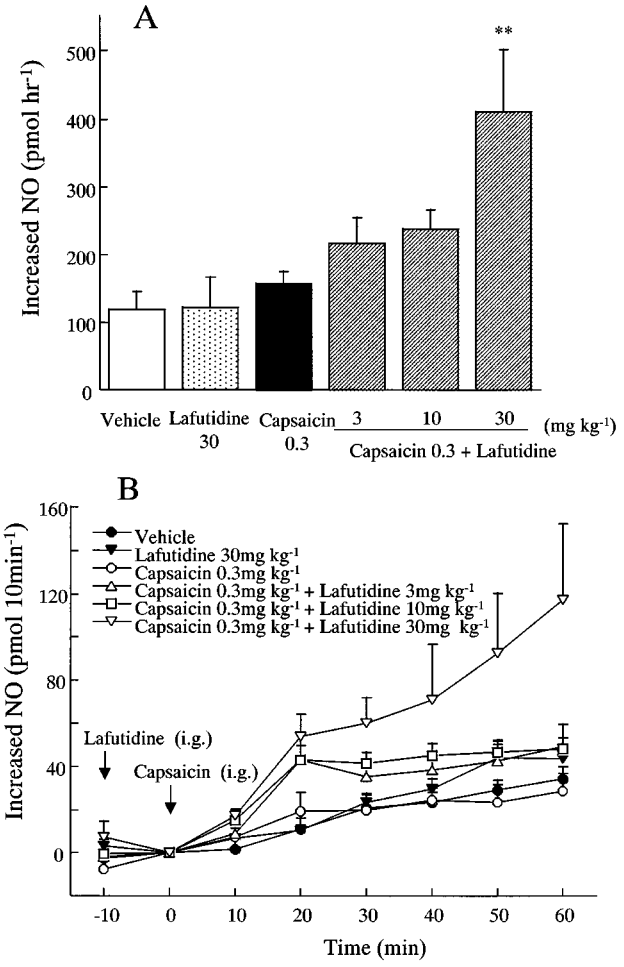

Figure 6 Effect of lafutidine on capsaicin-induced NO production from stomachs of anaesthetized rats. The perfused dialysates were collected from the stomach every 10 min and NO metabolites were determined with an automated NO detector HPLC system. (A) The sum of NO production over the basal level during 60 min period were calculated. (B) Changes in NO production in the dialysate. Capsaicin was intragastrically administered through a cannula in the forestomach and lafutidine was added 10 min before capsaicin treatment. Each point or column and vertical bar represents the mean and s.e.mean of 10 separate experiments. (A), $^{**}P<0.01$; significantly different from capsaicin (Dunnett's multiple comparison test).

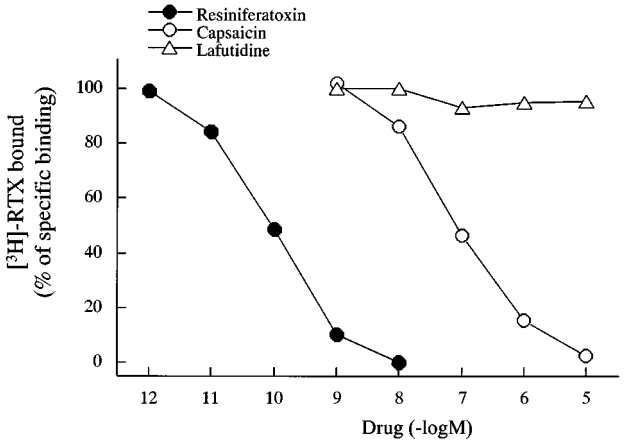

Figure 7 Effects of lafutidine, capsaicin and RTX on specific binding of [^{3}H]-RTX to rat gastric mucosal membranes. Specific binding of [^{3}H]-RTX was determined experimentally as the difference between total and nonspecific binding in parallel assays in the presence and absence of 1 μM RTX. One nM of [^{3}H]-RTX was incubated with five doses of each drug. All data were run in duplicate and each point represents the mean value of eight separate experiments.

with lafutidine, and 1.8 ± 0.1 nM and 143 ± 9 fmol mg^{-1} protein without lafutidine, respectively (Table 1). In addition, lafutidine (10^{-9}–10^{-5} M) did not influence the inhibitory effect of capsaicin (10^{-7} M) on [^{3}H]-RTX (1 nM) specific binding which produced 53% inhibition at this dose (data not shown). On [^{3}H]-RTX saturable binding study, the K_d and B_{max} values for [^{3}H]-RTX binding with capsaicin (10^{-7} M) were 1.9 ± 0.1 nM and 63 ± 2 fmol mg^{-1} protein with lafutidine (10^{-5} M), and 1.8 ± 0.1 nM and 61 ± 2 fmol mg^{-1} protein without lafutidine (10^{-5} M), respectively (Table 1).

Discussion

As shown in the results, capsaicin dose dependently increased CGRP release, while lafutidine alone had no effect on basal CGRP release from the rat stomach. On the other hand, lafutidine enhanced CGRP release induced by low dose capsaicin, which exerted a weak effect on CGRP release, and this potentiating effect was reversed by capsazepine treatment. In addition to its competitive antagonism of vanilloid receptors, capsazepine was reported to have a non-specific blocking action on voltage-activated calcium channels in rat cultured DRG cells (Docherty *et al*., 1997). Considering these factors, we suggested that lafutidine enhanced CGRP release by modulating signal stimulation through VR1 or signal transduction cascade related to calcium ion channels that may be linked to VR1. Although CGRP is reportedly located in cells other than nerves, gastric CGRP was reported to be located mainly in sensory neurons and to serve as the dominant neurotransmitter of spinal afferent nerves in the rat stomach (Green & Dockray, 1988; Jakab *et al*., 1993). Indeed, CGRP containing nerve fibres are immunostained around blood vessels in the submucosa and muscle layer of the rat stomach (Green & Dockray, 1988; Sternini *et al*., 1987), and staining was more intense in biopsy specimens from ulcer margins as compared to those from normal portions of the human stomach (Tani *et al*., 1999). In the physiological state, lafutidine would sensitize the CGRP containing sensory nerves when they are stimulated by back-diffused acid or other noxious chemicals in the stomach. Even in the setting of chronic ulcer, lafutidine would promote the healing process by sensitizing the intensely distributed afferent nerves.

In addition to its effects on CGRP release, capsaicin dose dependently increased NO production in the gastric wall, while lafutidine alone had no effect on the basal NO level. Lafutidine enhanced NO production with administration of a low dose of capsaicin which raised NO slightly above the basal level. Immunoreactive neuronal NO synthase (NOS) was mainly stained on the surface of the gastric mucosa, and endothelial NOS was detected in blood vessels in the lower region of the gastric glands and in the submucosa (Price & Hanson, 1998; Price *et al*., 1996). Furthermore neuronal NOS was also reported to be expressed in DRG neurons (Ren &

Table 1 Effect of lafutidine on saturation binding of [^{3}H]-RTX to rat gastric mucosal membranes

Agonist	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Control	1.8±0.1	143±9
With lafutidine (10^{-5} M)	1.7±0.1	135±8
With capsaicin (10^{-7} M)	1.9±0.1	63±2
With capsaicin (10^{-7} M) and lafutidine (10^{-5} M)	1.8±0.1	61±2

Specific binding of [^{3}H]-RTX was determined experimentally as the difference between total and nonspecific binding in parallel assays in the presence and absence of 1 μM RTX. Several concentrations of [^{3}H]-RTX were incubated with or without lafutidine (10^{-5}M) and capsaicin (10^{-7}M). All data were run in duplicate, and K_d and B_{max} values were estimated by Rosenthal analysis and presented as mean and s.e.mean of eight separate experiments.

Ruda, 1995; Vizzard *et al*., 1995), isolated epithelial mucus cells (Brown *et al*., 1992b; Byrne *et al*., 1997) and the plexus of Auerbach (Fischer *et al*., 1999) in the rat. In this study, although the origin of increased NO is not apparent, intravenous treatment with nitroprusside increased the NO content of the gastric wall and L-NAME decreased the basal NO level, suggesting the possibility of detecting, at least to some extent, NO from blood vessels. CGRP released from the sensory neurons was also reported to stimulate NO production through the $CGRP_1$ receptor on endothelial cells (Holzer, 1998). The NO produced participates in the regulation of gastric mucosal integrity by regulating gastric mucosal blood flow (GMBF) through vasodilation in the gastric microvasculature (Chen & Guth, 1995; Holzer *et al*., 1993), and also by secreting mucus or bicarbonate (Brown *et al*., 1992a; 1993). Furthermore, inhibition of NOS was reported to delay healing of chronic gastric ulcer (Konturek *et al*., 1993), and NO is also involved in the gastroprotective actions of CGRP (Lambrecht *et al*., 1993). NO may also be involved in modulating the activity of sensory neurons or the release of CGRP (Whittle, 1993). Additionally, intravenous and intragastric treatment with lafutidine was reported to elevate rat GMBF, an effect which was then attenuated by capsaicin induced defunctionalization of afferent nerves (Onodera *et al*., 1999a). Accelerated mucin biosynthesis of rat gastric mucosa, in an *in vitro* organ culture system, was blocked by N^G-nitro-L-arginine (Ichikawa *et al*., 1998). Lafutidine also accelerated recovery of the ammonia induced potential difference reduction in the rat stomach with a gastric chamber, which is closely responsible for the increase in GMBF and is attenuated by chemical defunctionalization of afferent nerves but not by indomethacin treatment (Onodera *et al*., 1999a). Therefore, lafutidine modulates the activities of gastric sensory nerves, and enhances CGRP and NO release that regulate the gastric mucosal microcirculation and maintains the mucosal integrity. The gastroprotective action of lafutidine, which was reversed by chemical defunctionalization of capsaicin sensitive afferent nerves, was observed even in the rats treatment with omeprazole (Onodera *et al*., 1995), and other H_2 receptor antagonists such as famotidine were reported to have little gastroprotective action against 100% ethanol, 0.6N HCl, 1% NH3, acidified sodium taurocholate or acidified ethanol induced rat gastric lesion (Onodera *et al*., 1995; Hoshino *et al*., 1991). In addition, famotidine (10^{-6} M) neither stimulated CGRP release nor potentiated capsaicin induced CGRP release from the rat stomach in our study (data not shown). From these reports and our data, it would appear that lafutidine's gastroprotective action and sensitizing effect on sensory neurons are independent of its antisecretory effect and its H_2 receptor blocking activity.

VR1, cloned recently (Caterina *et al*., 1997), is a ligand-gated, non-selective cation channel expressed mainly by sensory neurons (Caterina *et al*., 1997; Tominaga *et al*., 1998). VR1 is reportedly activated by noxious heat, acid and noxious stimuli including capsaicin, and may mediate thermal and chemical pain (Caterina *et al*., 1997; Davis *et al*., 2000; Tominaga *et al*., 1998). As for VR1 expression in the stomach, we previously showed VR1 immunopositive cells to be present in the rat gastric mucosal region, and specific [^{3}H]-RTX binding sites were found in this region (Nozawa *et al*., 2001). To explain the direct relation of lafutidine to afferent nerves, we next studied the VR1 binding profile in the stomach. In a [^{3}H]-RTX binding assay, although non-radiolabelled RTX and capsaicin competed with [^{3}H]-RTX for binding sites in the gastric mucosa, lafutidine showed no effect on [^{3}H]-RTX binding and similarly did not change K_d and B_{max} values of [^{3}H]-RTX specific binding. This suggested that lafutidine does not bind to the VR1 and does not modulate binding of a specific ligand to the VR1. Compared to the control value, there was a significant reduction of [^{3}H]-RTX binding sites (B_{max}) by treatment with capsaicin in rat stomach. In contrast, capsaicin had little effect on the K_d value for [^{3}H]-RTX binding. In another study, capsaicin was reported to reduced the K_d value of [^{3}H]-RTX binding with little effect on B_{max} value in rat DRG (Szallasi & Blumberg, 1999). Although we can not explain the differences between these results except by tissue differences, the predominant alteration in the density rather than in the apparent affinity of [^{3}H]-RTX binding site by capsaicin may be ascribable to its slowly dissociation from the receptors in rat stomach, as shown previously for the non-equilibrium blockade by calcium channel antagonists (Uchida *et al*., 1995; Yamada *et al*., 1992). In addition, in many *in vivo* studies, afferent nerves were reported to undergo irreversible loss of function with repeated administration of capsaicin (Buck & Burks, 1986), while daily administration of lafutidine did not decrease its gastroprotective actions (Onodera *et al*., 1995). From these results, we can assume that while capsaicin and lafutidine both function through afferent nerves, the two drugs have different mechanisms of action. Capsaicin directly stimulates VR1, and lafutidine sensitizes afferent sensory nerves through an unknown mechanism in the stomach.

In this study, we assessed the potentiating effects of lafutidine on the response to capsaicin. Although the mechanisms underlying this potentiation are not clear, we can speculate that lafutidine modifies intracellular signalling pathway after VR1 binding, or binds to another receptor related to the VR1 signalling pathway, or induces second messenger release from other tissues such as nervi nevorum, fibroblasts and gastric epithelial cells. Therefore, further studies, possibly using DRG neurons, are needed to clarify the potentiation mechanisms of lafutidine.

In summary, the present experiments demonstrated that (1) lafutidine enhanced CGRP and NO release from the rat stomach induced by a submaximal dose of capsaicin without

changing basal CGRP and NO release; and (2) lafutidine had no effect on specific [^{3}H]-RTX and capsaicin binding to VR1 in the rat stomach. These results suggest that lafutidine modulates the activity of capsaicin sensitive afferent nerves in the stomach, which may be a key mechanism underlying its gastroptorective action.

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