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Gastric antisecretory role and immunohistochemical localization of cannabinoid receptors in the rat stomach

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1 The role of cannabinoid (CB) receptors in the regulation of gastric acid secretion was investigated in the rat by means of functional experiments and by immunohistochemistry.

2 In anaesthetized rats with lumen-perfused stomach, the non selective CB-receptor agonist WIN 55,212-2 (0.30–4.00 μmol kg^{-1}, i.v.) and the selective CB_1-receptor agonist HU-210 (0.03–1.50 μmol kg^{-1}, i.v.), dose-dependently decreased the acid secretion induced by both pentagastrin (30 nmol kg^{-1} h^{-1}) and 2-deoxy-D-glucose (1.25 mmol kg^{-1}, i.v.). By contrast, neither WIN 55,212-2 (1–4 μmol kg^{-1}, i.v.) nor HU-210 (0.03–1.50 μmol kg^{-1}, i.v.) did modify histamine-induced acid secretion (20 μmol kg^{-1} h^{-1}). The selective CB_2-receptor agonist JWH-015 (3–10 μmol kg^{-1}, i.v.) was ineffective.

3 The gastric antisecretory effects of WIN 55,212-2 and HU-210 on pentagastrin-induced acid secretion were prevented by the selective CB_1-receptor antagonist SR141716A (0.65 μmol kg^{-1}, i.v.) and unaffected by the selective CB_2-receptor antagonist SR144528 (0.65–2 μmol kg^{-1}, i.v.).

4 Bilateral cervical vagotomy and ganglionic blockade with hexamethonium (10 mg kg^{-1}, i.v., followed by continuous infusion of 10 mg kg^{-1} h^{-1}) significantly reduced, but not abolished, the maximal inhibitory effect of HU-210 (0.3 μmol kg^{-1}, i.v.) on pentagastrin-induced acid secretion; by contrast, pretreatment with atropine (1 mg kg^{-1}, i.v.) did not modify the antisecretory effect of HU-210.

5 Immunoreactivity to the CB_1 receptor was co-localized with that of the cholinergic marker choline acetyltransferase in neural elements innervating smooth muscle, mucosa and submucosal blood vessels of rat stomach fundus, corpus and antrum. In contrast, CB_2 receptor-like immunoreactivity was not observed.

6 These results indicate that gastric antisecretory effects of cannabinoids in the rat are mediated by suppression of vagal drive to the stomach through activation of CB_1 receptors, located on pre- and postganglionic cholinergic pathways. However, the ineffectiveness of atropine in reducing the effect of HU-210 suggests that the release of non cholinergic excitatory neurotransmitters may be regulated by CB_1 receptors.

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Abbreviations: ANOVA, analysis of variance; ATR, atropine; BSA, bovine serum albumine; CB, cannabinoid; ChAT, choline acetyltransferase; CLSM, confocal laser scanning microscope; Cy3, indocarbocyanine; DMSO, dimethylsulphoxide; ECL, enterochromaffin-like; FITC, fluorescein isothiocyanate; HEX, hexamethonium; HU-210, 3-(1,1-dimethylheptyl)-(−)-11-hydroxy-Δ^8-tetrahydrocannabinol; IgG, immunoglobulin G; JWH-015, (1-propyl-2-methyl-3-(1-naphthoyl)indole); PBS, phosphate-buffered saline; PGP 9.5, protein gene product 9.5; Sham, sham vagotomy; SR141716A, N-(piperidin-1-y1)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; SR144528, N-[(1S)-endo-1,3,3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide); Δ^9-THC, Δ^9-tetrahydrocannabinol; VAG, vagotomy; WIN 55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-napthalenyl) methanone mesylate

Introduction

Over the past decade, there has been a growing interest in the pharmacology of cannabinoids. Two distinct cannabinoid (CB) receptors have been identified in mammalian tissues and selective agonists and antagonists have been discovered (Pertwee, 2000; 2001). Both CB_1 and CB_2 receptors are members of the superfamily of G-protein-coupled receptors; CB_1 receptors are primarily expressed in central and peripheral neurons and mediate the psychoactive effects of cannabinoids (Felder & Glass, 1998), whereas CB_2 receptors are predominantly located in peripheral tissues, mostly in immune cells and

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their physiological role has still to be established (Munro *et al*., 1993; Klein *et al*., 1998). In the gastrointestinal tract, CB_1 receptors have been involved in the control of intestinal motility in different animal species (Heinemann *et al*., 1999; Izzo *et al*., 2000) and in humans (Croci *et al*., 1998). Recently, cannabinoids have been found to reduce intestinal secretion (Tyler *et al*., 2000) and to exert gastroprotective effects against the damage induced by stress (Germanò *et al*., 2001), thus confirming previous data obtained with Δ^9-tetrahydrocannabinol (Δ^9-THC) in pylorus-ligated rats (Winn *et al*., 1976; Rivas-V & Garcia, 1980).

We have previously reported that two synthetic CB-receptor agonists, the aminoalkylindole WIN 55,212-2 and the tricyclic cannabinol derivative HU-210, significantly reduced pentagastrin-induced acid secretion in the anaesthetized rat, whereas the selective CB_2-receptor agonist JWH-015 had no effect (Coruzzi *et al*., 1999; Adami *et al*., 2000). In the present study, the mechanism underlying the gastric anti-secretory effects of the mixed CB_1/CB_2-receptor agonist WIN 55,212-2 and of the selective CB_1-receptor agonist HU-210 was investigated in the anaesthetized rat on the production of acid induced by direct stimuli of the oxyntic cells (histamine), or by indirect stimuli (2-deoxy-D-glucose and pentagastrin). The role of vagal cholinergic innervation was studied in rats pretreated with cervical vagotomy, ganglionic blockade or with atropine. Moreover, the localization of CB receptors in the rat gastric mucosa was investigated by means of immunohistochemistry.

A preliminary account of this study has been communicated to the British Pharmacological Society (Adami *et al*., 2001).

Methods

Effects of CB-receptor agonists and antagonists on gastric acid secretion in vivo

Male Wistar rats (200–250 g) were used. They were housed at constant temperature (20°C) and humidity (50–55%), with alternating 12-h light and dark cycles. Rats were fed standard laboratory chow and tap water. Gastric acid secretion was measured as described previously (Bertaccini *et al*., 1968). After urethane anaesthesia (1.25 g kg^{-1}, i.p.), the stomach was perfused at constant volume (60 ml h^{-1}) with saline (0.9% NaCl) at 37°C through an esophageal cannula. The perfusate was collected *via* a duodenal cannula and titrated to pH 7.0 at 10 min intervals with 10 mM NaOH using an automatic titrator system (Radiometer Copenhagen, Denmark).

After surgery, a period of 60 min was allowed for stabilization; once the gastric acid output had remained constant for 30 min, it was considered as basal acid secretion. Thereafter, to obtain submaximal stimulation of gastric acid output different secretagogues were administered in separate sets of experiments, either by continuous i.v. infusion (6 ml kg^{-1} h^{-1}) throughout the experiment (pentagastrin, 30 nmol kg^{-1} h^{-1} and histamine, 20 μmol kg^{-1} h^{-1}) or by rapid injection (2-deoxy-D-glucose, 1.25 mmol kg^{-1}, i.v.) into a tail vein. CB-receptor agonists were administered by i.v. bolus at the plateau of acid secretion induced by the different secretagogues. Control groups received the drug vehicle dimethylsulphoxide (DMSO) under the same conditions. Gastric acid response was monitored for 120 min after injection of cannabinoids or vehicle. To investigate the effect of CB-receptor antagonists, rats were pretreated with either the CB_1-receptor antagonist SR141716A or the CB_2-receptor antagonist SR144528, which were administered i.v. at the plateau of pentagastrin-induced secretion, starting 30 min before WIN 55,212-2 or HU-210 administration. In further experiments, the effects of CB-receptor antagonists alone were evaluated on pentagastrin-induced acid secretion.

Role of vagal innervation in gastric acid secretion stimulated by pentagastrin and in the inhibitory effect of HU-210

To investigate the role of the vagus nerve in the inhibitory action of CB-receptor agonists on gastric acid secretion, some rats underwent bilateral cervical vagotomy. Vagotomy was performed in anaesthetized animals by carefully dissecting the nerve and surrounding connective tissue from the carotid artery at a high cervical level. Then, both vagal nerves were firmly ligated and cut. In sham-operated animals, vagi were dissected from carotid arteries but not ligated or cut. Surgical dissection was carried out at the beginning of the experiment, but vagi were cut when the acid secretory response to pentagastrin had reached a stable plateau, since vagotomy performed at the beginning of the experiment did not produce reliable responses to pentagastrin. HU-210 or vehicle alone was administered 40–60 min after either vagotomy or sham operation. In another set of experiments, hexamethonium (10 mg kg^{-1} i.v. bolus, followed by 10 mg kg^{-1} h^{-1} continuous infusion), was administered at the plateau of pentagastrin-induced acid secretion, and HU-210 (0.3 μmol kg^{-1}, i.v.) was injected 40–60 min afterwards. In other experiments, atropine was given at 1 mg kg^{-1}, i.v. 40–60 min before HU-210 (0.3 μmol kg^{-1}, i.v.) injection. The atropine dose was selected by previously checking the effectiveness against the acid secretion induced by 2-deoxy-D-glucose (1.25 mmol kg^{-1}, i.v.).

Measurement of gastric acid secretion

Gastric acid output was expressed as absolute values in μEq HCl kg^{-1} min^{-1}. Acid responses to the different secretagogues were calculated for each rat by subtracting the average of two collection periods before the stimulant injection (basal acid output) from the peak acid secretion (mean of three different collection periods) obtained after the stimulant administration (Δ μEq HCl kg^{-1} min^{-1}). In order to determine agonist potency, the inhibitory effect of CB-receptor agonists was expressed as per cent inhibition of the stimulated-acid secretion (plateau response), that was considered arbitrarily as 100%. For each agonist, ED_{50} (dose which produced a 50% of the maximal possible effect) was derived from the inhibitory dose-response curves and reported with 95% confidence limits, together with E_{max} (maximal effect) values.

Localization of CB-receptor immunoreactivity in the rat stomach

Three adult Sprague-Dawley rats (300–350 g) were anaesthetised with isoflurane and were sacrificed by cervical

dislocation. Their stomachs were removed and the fundus, corpus and antrum were isolated by dissection and immersed in ice-cold 2% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 120 min. All tissues were cryoprotected in graded (10–30%) concentrations of sucrose in PBS, embedded and frozen in Tissue Tek O.C.T. compound (Baxter Healthcare Corp., McGaw Park, IL, U.S.A.). Coronal and longitudinal sections of fundus, corpus and antrum (15 μm thickness) were thaw-mounted onto Superfrost-plus™ slides (Fisher Scientific, Pittsburgh, PA, U.S.A.) and stored at −20°C until used.

To localize CB_1 receptor-like immunoreactivity in the stomach, antisera raised in rabbits against the N-terminus of the human CB_1 receptor were used. They were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.) and Biosource International (Camarillo, CA, U.S.A.). To examine the presence and distribution of CB_2-receptor immunoreactivity, a polyclonal antiserum raised in rabbits against amino acids 20–33 in the human CB_2 receptor was used; it was purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.).

To identify cholinergic neurons, a goat anti-choline acetyltransferase (ChAT) antibody was purchased from Chemicon International, Inc. (Temecula, CA, U.S.A.). Immunoreactivity to the general neuronal marker protein gene product 9.5 (PGP 9.5) was also examined using a rabbit anti-human PGP 9.5 antibody (Chemicon). Donkey anti-rabbit IgG-indocarbocyanine (Cy3)- and donkey anti-goat IgG-fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, U.S.A.). The corresponding peptides used in preabsorption control experiments for CB_1 receptors and ChAT were purchased respectively from Biosource International and Sigma Chemical Co. (St. Louis, MO, U.S.A.).

A standard immunofluorescence staining protocol was followed. Briefly, tissue sections were rehydrated in PBS (pH 7.4) for 15 min, then incubated in 0.4% Triton X-100 (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 2% bovine serum albumin (BSA, Sigma) in PBS for 30 min to block non-specific binding. Sections were simultaneously incubated in antibodies to CB_1 receptors (1 : 600 dilution) and ChAT (1 : 30 dilution) in 0.4% Triton X-100 and 2% BSA overnight at 4°C. After three rinses in PBS, sections were further incubated with appropriate secondary antibodies (donkey anti-rabbit Cy3-conjugated IgG at 1 : 400 dilution; or donkey anti-goat FITC-conjugated IgG at 1 : 40 dilution) in PBS for 60 min in the dark at 25°C. After three rinses in PBS for 15 min, coverslips were mounted with Vectashield™ (Vector Laboratory, Burlingame, CA, U.S.A.) and the edges sealed with nail polish. Antibody to PGP 9.5 (1 : 150 dilution) was used as general neuronal marker to confirm neuronal morphology, adjacent sections were incubated with antibody to PGP 9.5 overnight at 4°C. Additional steps were carried out as described above.

Controls consisted of omission of the primary antibody from the staining protocol; replacing the primary antibody with another unrelated primary antibody; or preabsorbing the primary antibody against the antigen that resulted in complete absence of specific immunoreactivity. Similar distribution patterns of CB_1-receptor immunoreactivity were observed using both anti-CB_1-receptor antisera obtained from two different commercial sources. However, only the antibody obtained from Biosource International could be tested in preabsorption experiments because a blocking peptide for the antibody purchased from Cayman Chemicals was not available. The CB_1-receptor peptide fragment in 100 molar excess and 1–2 μg ml^{-1} of ChAT was incubated overnight at 4°C with the relevant primary antibody to preabsorb the antisera. After centrifugation, the supernatant was used in place of the primary antibody in the staining protocol. Preincubation of CB_1-receptor antiserum with 100 molar excess of CB_1-receptor peptide N-terminal fragment completely eliminated the ability of the antibody to detect immunoreactive neural elements in any gastric subregion.

Tissue sections were scanned using a BioRad confocal laser-scanning microscope (CLSM; Model 1024) which was attached to a Nikon fluorescence microscope. Images were obtained using Comos software (version 6.05.8; Comos BioRad, Hercules, CA, U.S.A.) and further processed employing NIH Image (version 1.59) and Adobe Photoshop (version 4.0) software.

Statistical analysis

Values have been expressed as means and variability as s.e.mean or as 95% confidence limits. Comparisons between multiple groups were made by using one-way analysis of variance (ANOVA), followed by Newman-Keuls test. Values of $P<0.05$ were considered as statistically significant. The software package Prism GraphPad (version 3.0) was used to perform the analyses.

Drugs

Atropine, 2-deoxy-D-glucose, hexamethonium, histamine and pentagastrin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). WIN 55,212-2 *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate was from RBI (Natick, MA, U.S.A.). HU-210 3-(1,1-dimethylheptyl)-(−)-11-hydroxy-Δ^8-tetrahydrocannabinol was from Tocris Cookson (Ballwin, MO, U.S.A.). SR141716A N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride and SR144528 N-[(1S)-endo-1,3,3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methyl phenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) were kindly supplied by Dr Madeleine Mossé (Sanofi Recherche, Montpellier, France). JWH-015 (1-propyl-2-methyl-3-(1-naphthoyl) indole) was a generous gift from Dr John W. Huffman (Department of Chemistry, Clemson University, Clemson, SC, U.S.A.). Atropine, 2-deoxy-D-glucose, hexamethonium and histamine were dissolved in distilled water. All the other compounds were dissolved in 100% DMSO.

Results

Effect of CB-receptor agonists and antagonists on gastric acid secretion in vivo

In anaesthetized rats with lumen-perfused stomach WIN 55,212-2 (0.3–4 μmol kg^{-1}, i.v.) and HU-210 (0.03–1.5 μmol kg^{-1}, i.v.), caused a dose-dependent reduction of

Table 1 ED_{50} and E_{max} values of WIN 55,212-2 and HU-210 on pentagastrin- or 2-deoxy-D-glucose-induced acid secretion in the anaesthetized rat with lumen-perfused stomach

	Pentagastrin		*2-deoxy-D-glucose*	
Drug	ED_{50} (μmol kg^{-1}, i.v.)	E_{max}	ED_{50} (μmol kg^{-1}, i.v.)	E_{max}
WIN 55,212-2	1.24 (0.24–6.30)	66.30±11.50[a]	1.99 (0.62–6.55)	51.62±14.50[a]
HU-210	0.11 (0.04–0.35)	100	0.23 (0.08–0.65)	93.22±6.78

Values are shown with 95% confidence limits (ED_{50}) or s.e.mean (E_{max}). E_{max} = maximal per cent decrease of pentagastrin- or 2-deoxy-D-glucose-induced acid secretion, considered as 100. [a]E_{max} values for WIN 55,212-2 refer to the dose of 2 μmol kg^{-1}, i.v. Higher doses caused death of the animals (see text).

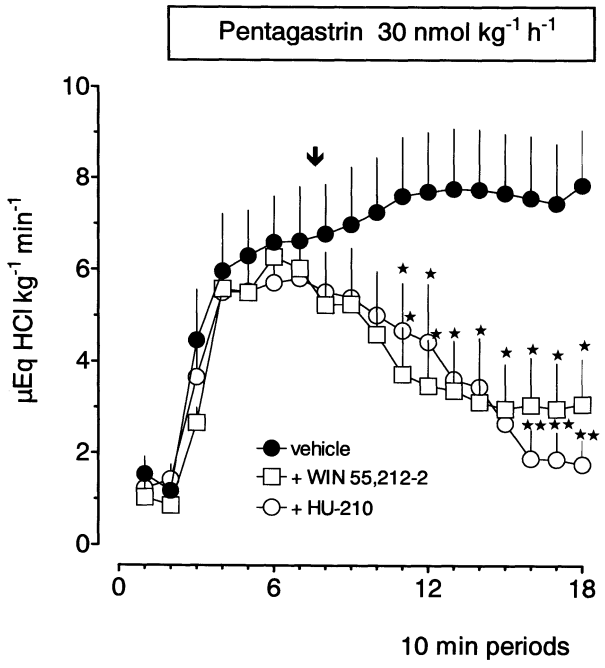

Figure 1 Anaesthetized rat with lumen-perfused stomach. Time-course of the inhibitory effects of equiactive doses of WIN 55,212-2 (2 μmol kg^{-1}) and of HU-210 (0.3 μmol kg^{-1}) administered (↓) i.v. at the plateau of the acid secretion induced by continuous i.v. infusion of pentagastrin. In control experiments, the drug vehicle (DMSO, 0.1 ml rat^{-1}, i.v.) was administered instead of CB-receptor agonists. Values represent the mean±s.e.mean of responses in 6–8 animals for each experimental group. *$P<0.05$ and **$P<0.01$ *vs* vehicle.

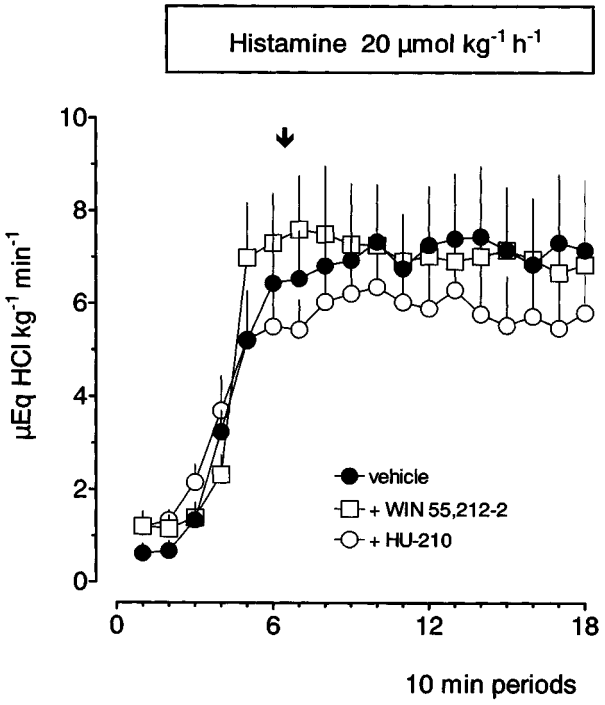

Figure 2 Anaesthetized rat with lumen-perfused stomach. Effects of WIN 55,212-2 (4 μmol kg^{-1}) and HU-210 (1.5 μmol kg^{-1}) administered (↓) i.v. at the plateau of the acid secretion induced by continuous i.v. infusion of histamine. In control experiments, the drug vehicle (DMSO, 0.1 ml rat^{-1}, i.v.) was administered instead of CB-receptor agonists. Values represent the mean±s.e.mean of responses in 7–9 animals for each experimental group.

the acid secretion induced by either pentagastrin (30 nmol kg^{-1} h^{-1}) or 2-deoxy-D-glucose (1.25 mmol kg^{-1}, i.v.) (Table 1). The time-course of the inhibitory effects of equiactive doses of WIN 55,212-2 (2 μmol kg^{-1}, i.v.) and of HU-210 (0.3 μmol kg^{-1}, i.v.) on pentagastrin-induced acid secretion is shown in Figure 1. HU-210 was approximately 10 times more potent than WIN 55,212-2, as revealed by the ED_{50} values, and behaved as a full agonist against either secretagogues (Table 1). It was not possible to determine the maximum effect of WIN 55,212-2, since four out of five animals and three out of four animals died when the highest dose of WIN 55,212-2 (4 μmol kg^{-1}, i.v.) was administered against pentagastrin- or 2-deoxy-D-glucose, respectively. No animal died following the highest dose tested (1.5 μmol kg^{-1}, i.v.) of HU-210. Neither WIN 55,212-2 (1–4 μmol kg^{-1}, i.v.) nor HU-210 (0.03–1.5 μmol kg^{-1}, i.v.) did modify histamine-induced acid secretion (Figure 2). The selective CB_1-receptor antagonist SR141716A (0.65 μmol kg^{-1}, i.v.) did not significantly change the gastric acid secretion induced by pentagastrin, but prevented the inhibitory effects of WIN 55,212-2 (2 μmol kg^{-1}, i.v.) and HU-210 (0.3 μmol kg^{-1}, i.v.) (Figure 3). SR141716A, administered i.v. at 2 μmol kg^{-1}, significantly reduced (45.6±8.4%, $P<0.05$) pentagastrin-induced acid secretion, while leaving unaltered the acid secretion induced by histamine (data not shown). The selective CB_2-receptor antagonist SR144528 (0.65–2 μmol kg^{-1}, i.v.) was inactive either on pentagastrin-induced acid secretion or on the inhibitory effect of HU-210 (0.3 μmol kg^{-1}, i.v., Figure 4).

The selective CB_2-receptor agonist JWH-015 (3–10 μmol kg^{-1}, i.v.) was ineffective on basal acid secretion and on the acid response to pentagastrin and 2-deoxy-D-glucose (data not shown).

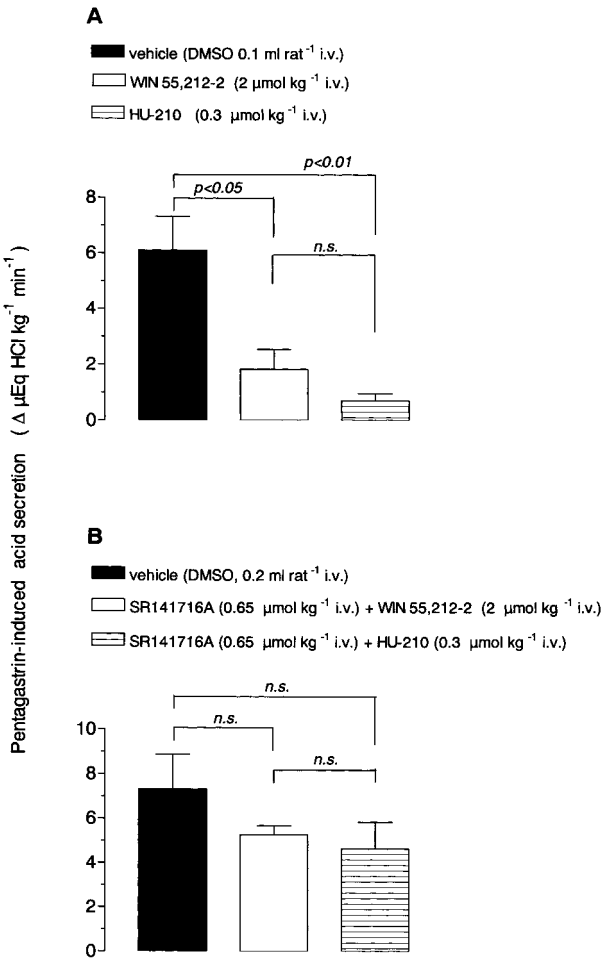

Figure 3 Anaesthetized rat with lumen-perfused stomach. (A) Effect of WIN 55,212-2 and HU-210 on pentagastrin-induced acid secretion (30 nmol kg^{-1} h^{-1}). (B) Effect of SR141716A and SR144528 on the inhibitory action of either WIN 55,212-2 or HU-210 on pentagastrin-induced acid secretion (30 nmol kg^{-1} h^{-1}). In control experiments, the drug vehicle was administered instead of CB-receptor ligands. Values represent the mean ± s.e.mean of responses in 6–8 animals for each experimental group. n.s. = not significant.

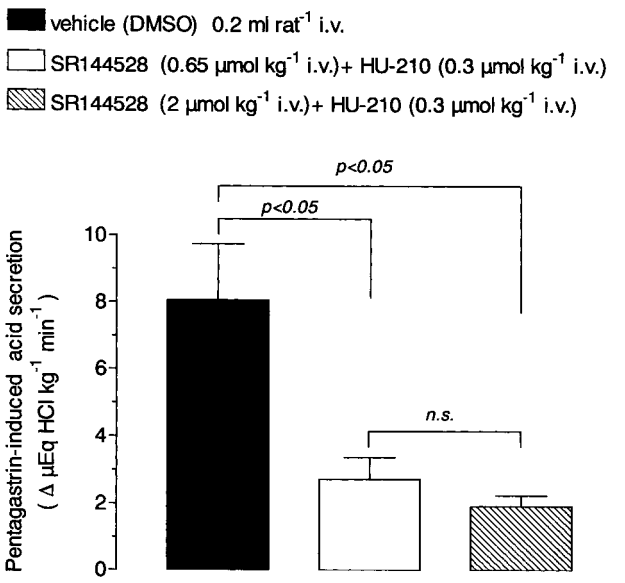

Figure 4 Anaesthetized rat with lumen-perfused stomach. Effect of SR144528 on the inhibitory action of HU-210 on pentagastrin-induced acid secretion (30 nmol kg^{-1} h^{-1}). In control experiments the drug vehicle was administered instead of CB-receptor ligands. Values represent the mean ± s.e.mean of responses in 6–8 animals for each experimental group. n.s. = not significant.

Effect of vagotomy, hexamethonium or atropine on the acid secretion stimulated by pentagastrin and on the inhibitory effect of HU-210

The involvement of cholinergic pathways in the CB-receptor agonist-induced inhibition of acid secretion was evaluated in rats pretreated with cervical vagotomy, hexamethonium or atropine. In these animals the effect of HU-210 was evaluated against pentagastrin-induced acid secretion. Results showed that bilateral cervical vagotomy significantly reduced the acid response to pentagastrin and the inhibitory action of HU-210 (0.3 μmol kg^{-1}, i.v., Figure 5). Hexamethonium (10 mg kg^{-1}, i.v. bolus administered at the top of pentagastrin-induced acid secretion, followed by infusion of 10 mg $kg^{-1}h^{-1}$), did not significantly modify pentagastrin response, while reducing the inhibitory effect of HU-210 (0.3 μmol kg^{-1}, i.v., Figure 5). By contrast, atropine (1 mg kg^{-1}, i.v.) did not significantly change the acid response to pentagastrin and the effect of HU-210 (0.3 μmol kg^{-1}, i.v., Figure 5), while abolishing the acid response to 2-deoxy-D-glucose (1.25 mmol kg^{-1}, i.v., data not shown).

Localization of CB receptor-like immunoreactivity in rat stomach

Intense CB_1-receptor immunoreactivity was observed in fundus, corpus and antrum; in contrast CB_2-receptor immunoreactivity was not observed in any region of the stomach. Some differences were noted in the pattern of CB_1-receptor immunoreactivity. In the fundus, intense CB_1-receptor immunoreactivity was localized in neurons within the myenteric plexus. In most of these neurons, CB_1-receptor immunoreactivity was highly colocalized with immunoreactivity to the cholinergic marker, ChAT (Figure 6A–A1). Numerous fibres co-expressing CB_1-receptor and ChAT immunoreactivities were observed in the circular muscle; in some fibres however, only CB_1-receptor immunoreactivity was seen (Figure 6A1). Immunoreactivities to the CB_1 receptor and ChAT were also colocalized in the neurons situated in ganglia subjacent to the gastric epithelium (Figure 6A2). In many cases, a diffuse pattern of CB_1-receptor immunoreactivity was observed in neurons and some fibers. CB_1-receptor and ChAT immunoreactivities were observed in thick-walled blood vessels in the submucosa, and in some cases, at the inner lining of the blood vessel, a region probably corresponding to the endothelium (Figure 6A3).

In the gastric corpus, CB_1-receptor and ChAT immunoreactivities were not as highly colocalized in neurons or nerve fibres as they were in the fundus (Figure 6B). However, weak ChAT immunoreactivity was observed in several CB_1-receptor immunoreactive neurons in the myenteric plexus (Figure 6C). Similarly, CB_1-receptor immunoreactivity was observed in both ChAT-positive and -negative submucosal

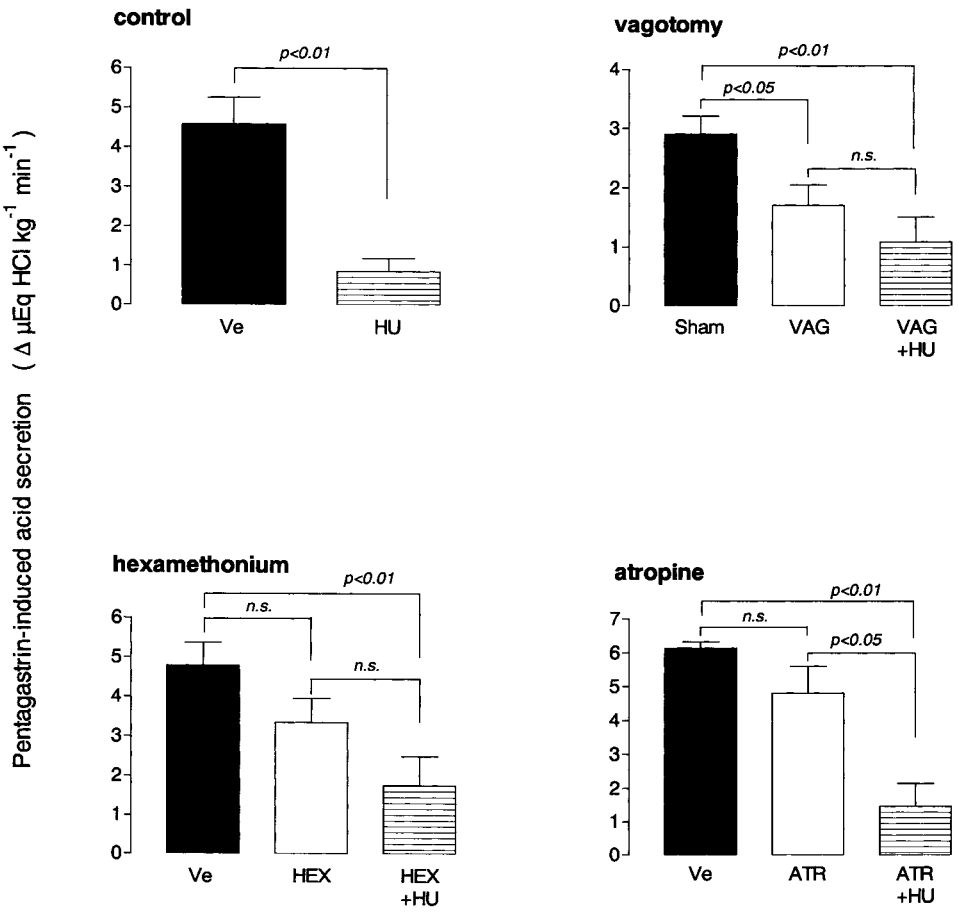

Figure 5 Anaesthetized rat with lumen-perfused stomach. Effect of vagotomy (VAG), sham vagotomy (Sham), hexamethonium (HEX, 10 mg kg^{-1} i.v. bolus, followed by 10 mg kg^{-1} h^{-1} continuous infusion), atropine (ATR, 1 mg kg^{-1} i.v.) or vehicle (Ve, DMSO 0.2 ml rat^{-1} i.v.) on the acid response to pentagastrin in the absence or in the presence of HU-210 (HU, 0.3 μmol kg^{-1}, i.v.). Values represent the mean$\pm$s.e.mean of responses in 6–8 animals for each experimental group. n.s. = not significant.

nerve fibres. No CB_1-receptor immunoreactivity was observed around submucosal blood vessels or in submucosal neurons.

In contrast to the fundus and corpus, the antrum contained large myenteric neurons which co-contained CB_1-receptor and ChAT immunoreactivities (Figure 6D). However, nerve fibres terminating in the smooth muscle layers exhibited immunoreactivity for CB_1 receptors, but not for ChAT. In the submucosa, there was an absence of CB_1-receptor immunoreactive nerve fibres, although some ChAT immunoreactive fibres could be seen (Figure 6E).

Discussion

The present study showed that CB_1 receptors mediate the inhibitory effects of CB-receptor agonists on the acid secretion induced by pentagastrin and 2-deoxy-D-glucose in the anaesthetized rat. These data confirm and extend previous studies indicating that CB-receptor agonists reduced the secretory effects of single administrations of pentagastrin (Coruzzi *et al*., 1999; Adami *et al*., 2000). The two agonists employed in this study, namely WIN 55,212-2 and HU-210, behaved similarly in reducing pentagastrin and 2-deoxy-D-glucose, being HU-210 approximately 10 times more potent than WIN 55,212-2. This last compound was not well tolerated when administered at doses higher than 2 μmol kg^{-1}, because of intense bradycardia and hypotension. Serious cardiovascular effects have been frequently associated with CB_1-receptor agonist administration (Graham & Li, 1973; Lake *et al*., 1997). However, since the toxic effects were observed with the mixed CB_1/CB_2-receptor agonist WIN 55,212-2 but not with the more selective CB_1 agonist HU-210, the involvement of CB_2 receptors cannot be definitely ruled out (Griffin *et al*., 1997).

The specific involvement of the CB_1-receptor subtype in the gastric antisecretory effect of CB-receptor agonists is suggested by several factors: (1) the activity of the selective agonist HU-210; (2) the antagonism induced by the selective CB_1-receptor antagonist SR141716A (Rinaldi-Carmona *et al*., 1995); and (3) the lack of effect of the CB_2-receptor agonist JWH-015 and the CB_2-receptor antagonist SR144528. As for the mechanism underlying the antisecretory effect of CB-receptor agonists, the reduction of the acid secretion induced by 2-deoxy-D-glucose and pentagastrin would suggest a location of CB_1 receptors on cholinergic pathways innervating parietal cells. In fact, 2-deoxy-D-glucose stimulates acid secretion by increasing efferent activity in gastric vagus nerve *via* glucopenia in hypothalamic neurons (Colin-Jones & Himsworth, 1970); on the other hand, pentagastrin-induced acid secretion is reported to involve both histaminergic and

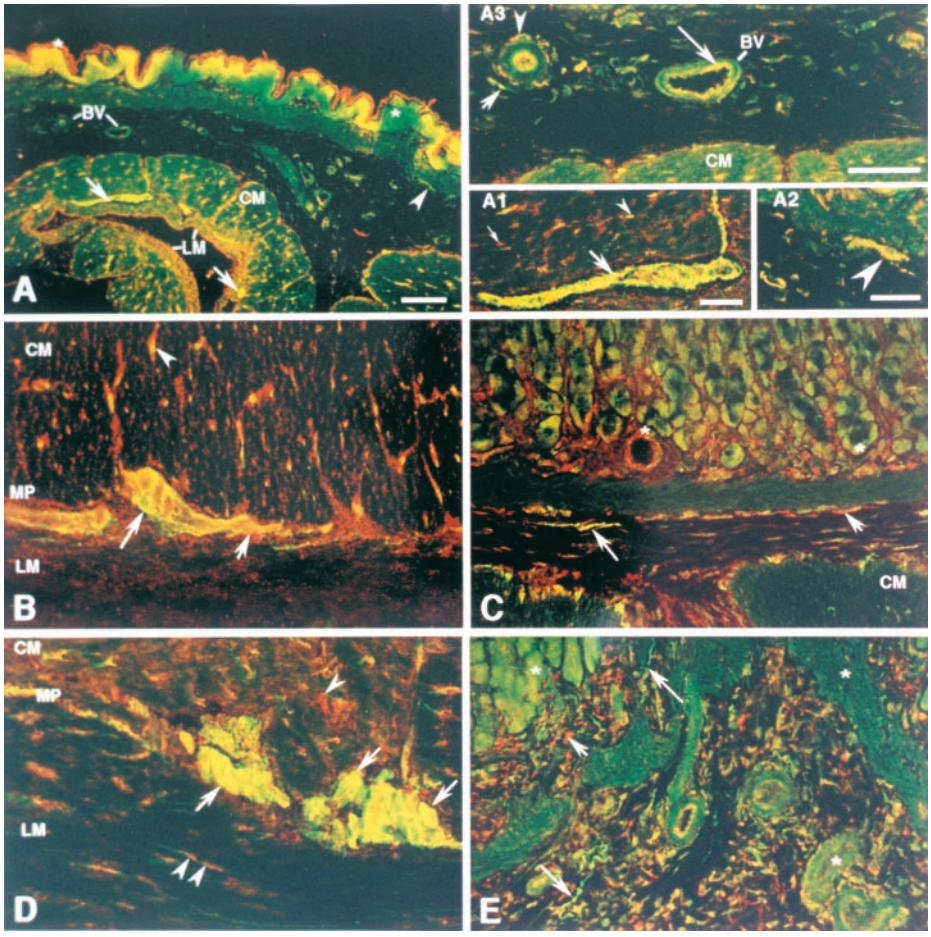

Figure 6 Localization of CB_1-receptor and ChAT immunoreactivity in fundus (A, A1, A2 and A3), corpus (B,C) and antrum (D,E) of rat stomach. (A) Low power micrograph of a longitudinal section through fundus. CB_1-receptor (red) and ChAT (green) immunoreactivities are colocalized (yellow) in myenteric neurons (arrow). Nonspecific labelling (*) persisted in preabsorption and omission controls. (A1) High power micrograph of the myenteric neurons seen in A (large arrow). Fibres can be observed which exhibit CB_1-receptor immunoreactivity alone (small arrow, red) or in combination with ChAT immunoreactivity (arrowhead, yellow). (A2) A single submucosal neuron exhibiting immunoreactivities to both CB_1 receptors and ChAT. Due to the diffuse nature of the CB_1-receptor immunoreactivity, a greenish hue is imparted to some of the neurons. (A3) CB_1-receptor and ChAT immunoreactivities are colocalized in nerve fibres innervating thick-walled blood vessels (short arrow and arrow head). CB_1-receptor and ChAT immunoreactivities were co-localized in fibres innervating the inner wall of the blood vessel (long arrow). (B) Myenteric neurons exhibiting CB_1-receptor immunoreactivity in the corpus (short arrow). In some neurons, CB_1-receptor immunoreactivity was colocalized with ChAT (large arrow). Most fibres terminating in the circular muscle exhibited CB_1-receptor immunoreactivity (red), but little ChAT immunoreactivity (yellow, arrowhead). (C) In the corpus, distinct CB_1-receptor-immunoreactive (short arrow) nerve fibres and fibres exhibiting both CB_1-receptor and ChAT immunoreactivity (yellow hue, long arrow) can be seen in the circular muscle layer. Some nonspecific labelling (*) is observed that persisted in omission and preabsorption controls. (D) Colocalization of CB_1-receptor and ChAT immunoreactivities in myenteric neurons (arrow) of the gastric antrum. Due to the diffuse nature of the CB_1-receptor immunoreactivity, a greenish hue is imparted to some of the neurons. CB_1-receptor-immunoreactive nerve fibres (double arrowhead) can be observed terminating in longitudinal muscle. Some nonspecific labelling (single arrow) persisted in omission and preabsorption controls. (E) In the antral submucosa, fibres immunoreactive for ChAT (green, long arrows) could be observed, but no CB_1-receptor-immunoreactive fibres were found. Some nonspecific labelling (short arrow) is observed that persisted in omission and preabsorption controls. Bar in A = 50 μm; bars in A1, B–E = 16 μm; bar in A2 = 13 μm; bar in A3 = 25 μm.

cholinergic pathways (Lin & Evans, 1970; Hakanson *et al*., 1982; Ekelund *et al*., 1987; Watanabe *et al*., 1996). Indeed, in our study vagotomy completely suppressed the acid response to 2-deoxy-D-glucose and significantly reduced that induced by pentagastrin. The reduction of the antisecretory effect of HU-210 induced by bilateral cervical vagotomy or ganglionic blockade suggests that the mechanism underlying inhibition of acid secretion by HU-210 is at least partly mediated by suppression of the vagal drive to the stomach, by an action at preganglionic sites. Peripherally injected CB-receptor agonists can easily cross the blood–brain barrier (Petitet *et al*., 1999) and could therefore reduce acid secretion by reducing vagal efferent activity at central sites. CB_1 receptors are widely expressed in central neurons and, in particular, in the nucleus dorsalis nervi vagi (Mailleux & Vanderhaeghen, 1992; Matsuda *et al*., 1993). Furthermore, inhibitory effects of CB-receptor agonists on intestinal motility are mediated by both central and peripheral CB_1 receptors (Izzo *et al*., 2000). However, previous data from our laboratory showed that neither HU-210 nor WIN 55,212-2, administered intracer-

ebroventricularly, were able to modify pentagastrin-induced acid secretion (Adami *et al*., 2001). It is well known, however, that centrally-administered drugs can differently affect gastric acid secretion, depending on the brain area of injection (Tachè, 1987). Therefore, it is possible that CB-receptor agonists, administered in different brain regions (nucleus dorsalis nervi vagi, lateral hypothalamus) can have opposite effects in the regulation of vagal efferent pathways and consequently in the production of gastric acid output.

The distribution of CB_1-receptor immunoreactivity in the rat stomach strongly suggests that peripherally located CB_1 receptors also contribute to the inhibitory effects observed in functional experiments. CB_1-receptor immunoreactivity, in fact, was detected in neural elements innervating smooth muscle, mucosa and submucosal blood vessels of rat stomach fundus, corpus and antrum using antisera directed against N-terminal epitopes in the human CB_1 receptor. Receptor-like immunoreactivity was often colocalized with immunoreactivity to choline acetyltransferase, a marker of cholinergic neurons, as recently observed in cholinergic neurons of the porcine enteric nervous system (Kulkarni-Narla & Brown, 2000). However, based on the ineffectiveness of atropine in reducing the antisecretory effect of HU-210, we hypothesize that CB_1 receptors can modulate the release of non cholinergic (peptidergic) neurotransmitters, located on mucosal intrinsic cholinergic fibres, such as vasoactive intestinal peptide, gastrin releasing peptide and pituitary adenylate cyclase activating peptide (Ekblad *et al*., 2000).

The lack of effect of WIN 55,212-2 and HU-210 on histamine-stimulated acid secretion, when tested at doses maximally effective against pentagastrin, excludes the possibility that the inhibitory effect of these compounds is due to a direct action on parietal cells. However, a location of CB_1 receptors on enterochromaffin-like (ECL) cells in the gastric mucosa, with the function of reducing histamine release, cannot be ruled out by the present experiments. Both pentagastrin (Hills *et al*., 1996; Prinz *et al*., 1999) and 2-deoxy-D-glucose (Ikarashi *et al*., 2000) can release endogenous histamine from gastric stores; therefore, a reduction of this histaminergic component by CB-receptor agonist can be hypothesized, when considering that the inhibitory effect of HU-210 was only partially reduced by vagotomy.

Immunoreactivity to CB_2 receptors was not observed in any region of the stomach using an antiserum raised against amino acids 20–33 in the human CB_2 receptor, suggesting that CB_2 receptors are not expressed in this organ. In this connection, previous data (Coruzzi *et al*., 1999) and the present study revealed that the selective CB_2-receptor agonist JWH-015 (Huffman *et al*., 1996) did not alter basal or stimulated acid secretion, suggesting a minor role of this receptor subtype. However, the CB_2-receptor protein exhibits relatively greater sequence divergence among species than does the CB_1-receptor and the failure of the anti-CB_2-receptor antiserum to detect receptor-like immunoreactivity in the stomach may be due to the 71% sequence homology between rat and human CB_2 receptors in the region detected by the antiserum (Griffin *et al*., 2000). On the basis of the present data with its attendant caveats, it is premature to conclude that this CB-receptor type is not involved in the modulation of gastric function.

In summary, the present results confirm that cannabinoids inhibit gastric acid secretion through interactions with CB_1 receptors. The inhibition of indirectly acting secretagogues, such as 2-deoxy-D-glucose and pentagastrin, together with the lack of effect against histamine-induced acid secretion, indicate that inhibitory CB_1 receptors are not expressed by parietal cells. Indeed, functional experiments with hexamethonium and cervical vagotomy together with immunohistochemical data suggest a predominant location of CB_1 receptors on vagal pathways to the gastric mucosa.

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