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Long-lasting cholecystokinin$_2$ receptor blockade after a single subcutaneous injection of YF476 or YM022

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1 Histamine-forming ECL cells in the rat stomach operate under the control of gastrin. They represent a convenient target for studying cholecystokinin-B/gastrin (CCK_2) receptor antagonists *in vivo*.

2 We examined the effectiveness and duration of action of two CCK_2 antagonists, YM022 and YF476, with respect to their effect on ECL-cell histidine decarboxylase (HDC) activity in the rat.

3 Oral administration of subcutaneous deposition of YF476 or YM022 reduced the HDC activity. The maximum/near-maximum dose for both drugs and for both modes of administration was 300 μmol kg^{-1} (effects measured 24 h after dose). At this dose and time the serum concentration of YF476 was 20–40 nmol l^{-1}. The dose 300 μmol kg^{-1} was used in all subsequent studies.

4 A single subcutaneous injection of YF476 inhibited the HDC activity for 8 weeks. The circulating concentration of YF476 remained high for the same period of time ($\geqslant$15 nmol l^{-1}). Subcutaneous YM022 suppressed the HDC activity for 4 weeks. A single oral dose of YF476 or YM022 inhibited the HDC activity for 2–3 days.

5 Chronic gastric fistula rats were used to study the effect of subcutaneous YF476 on gastrin-stimulated acid secretion. A single injection of YF476 prevented gastrin from causing an acid response for at least 4 weeks (the longest time studied).

6 We conclude that a single subcutaneous injection of 300 μmol kg^{-1} YF476 causes blockade of CCK_2 receptors in the stomach of the rat for 8 weeks thus providing a convenient method for studies of the consequences of long-term CCK_2 receptor inhibition.
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Keywords: CCK_2 receptor antagonist; ECL cell; histidine decarboxylase (HDC); acid output; gastrin

Abbreviations: CCK, cholecystokinin; HDC, histidine decarboxylase

Introduction

Gastrin and cholecystokinin (CCK) share the C-terminal pentapeptide amide sequence which carries the biological message. The two hormones interact with two distinct receptors, referred to as CCK-A (CCK_1) and CCK-B/gastrin (CCK_2). These receptors have been identified by cloning experiments (Kopin *et al*., 1992; Wank *et al*., 1992a,b; 1996) and characterized by binding studies (Innis *et al*., 1980; Beinfeld *et al*., 1983; Chang *et al*., 1989).

The ECL cells, which are histamine- and chromogranin A-producing endocrine/paracrine cells, are numerous in the acid-producing part of the stomach. In the rat, they make up 65–70% of all endocrine cells in this location (Håkanson *et al*., 1992; Nissinen & Panula, 1993). They operate under the control of circulating gastrin (Håkanson *et al*., 1992; 1993; 1994), which stimulates their secretory activity, protein synthesis rate and growth (Håkanson *et al*., 1992; 1993; 1994; Prinz *et al*., 1993; Chen *et al*., 1994). ECL-cell histamine plays a pivotal role in gastrin-stimulated acid secretion (Waldum *et al*., 1991; Black, 1993; Andersson *et al*., 1996). In the absence of ECL-cell histamine, gastrin is no longer capable of stimulating acid secretion (Andersson *et al*., 1996). Moreover, there is compelling evidence that the target cells for gastrin – the ECL cells – are furnished with CCK_2 receptors (Roche *et al*., 1991a,b; Sandvik & Waldum, 1991; Prinz *et al*., 1993; 1994; Asahara, 1994; Ding *et al*., 1995; 1997a,b; Ding & Håkanson, 1996a,b; Lindström *et al*., 1999).

YM022 (Nishida *et al*., 1994; Ding & Håkanson, 1996a) and YF476 (Semple *et al.,* 1997; Takinami *et al.,* 1997) are potent and selective CCK_2 receptor antagonists (Ding *et al*., 1997c) of the benzodiazepine type. They bind with 500–1000 fold higher affinity to CCK_2 receptors than to CCK_1 receptors (Nishida *et al*., 1994; Semple *et al*., 1997). They inhibit gastrin-induced gastric acid secretion in anaesthetized rats and Heidenhain pouch dogs (Semple *et al*., 1997; Takinami *et al*., 1997) and prevent gastrin-evoked ECL cell activation (histidine decarboxylase (HDC) activation and pancreastatin release) in the awake rat (Ding *et al*., 1997c). In the present report we investigate the pharmacological properties of YM022 and YF476 with respect to effectiveness and duration of action after subcutaneous or oral administration to conscious rats. The functional activity of the ECL cells in the rat stomach is reflected in the oxyntic mucosal HDC activity (Håkanson *et al*., 1992; 1993; 1994; Chen *et al*., 1994), which therefore was chosen as the parameter to be studied. Blockade of acid secretion leads to hypergastrinemia because of abolished acid feed-back inhibition of gastrin release. Consequently the serum gastrin concentration was used as another parameter to monitor drug-induced acid inhibition.

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Methods

Chemicals

YF476, (R)-1-[2,3-dihydro-2-oxo-1-pivaloylmethyl-5-(2-pyridyl)-1H-1, 4-benzo-diazepin-3-yl]-3-(3-methylaminophenyl)urea, was generously supplied to Dr. Alan Harris, Ferring A/S (Vanløse, Denmark). YM022 {(R)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1H-1, 4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea} was a gift from Dr K. Miyata, Yamanouchi Pharmaceutical (Ibaraki, Japan). Synthetic rat gastrin-17 (pGlu-Arg-Pro-Pro-Met-Glu-Glu-Glu-Glu- Glu- Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2) was obtained from Research Plus (Bayonne, NJ, U.S.A.). YF476 and YM022 were suspended in 2% Methocel (Dow Corning, Midland, MI, U.S.A.) for oral ingestion and in polyethylene glycol 300 (Acros Organics, Springfield, NJ, U.S.A.) for subcutaneous injection. Gastrin-17 was dissolved in 0.9% saline enriched with 1% bovine serum albumin in order to reduce adsorption to surfaces.

Animals

Male Sprague-Dawley rats, weighing 200–250 g, were kept in plastic cages (5–6 rats in each cage) with free access to standard rat food pellets (Lactamin, Vadstena, Sweden) and tap water throughout the study (if not otherwise stated). At the end of each experiment, the rats were killed (between 1000 and 1200 h) by exsanguination from the abdominal aorta (chloral hydrate anaesthesia, 300 mg kg^{-1} by the intraperitoneal route). Serum was collected and stored at $-20^{\circ}C$ until determination of gastrin by radioimmunoassay and YF476 by HPLC (see below). After weighing the animals, the stomachs were removed, opened along the major curvature and rinsed with ice-cold 0.9% saline. For comparison, 10 rats were deprived of food (but not water) for 48 h and killed. The oxyntic mucosa was collected, weighed and stored at $-20^{\circ}C$ until analysed for histidine decarboxylase activity.

In some experiments, gastric acid secretion in response to gastrin was studied in rats fitted with a chronic gastric fistula. The fistula was implanted in the rumen close to the glandular part of the stomach (Bel *et al*., 1996) under chloral hydrate anaesthesia. A recovery period of 2 weeks was allowed before the secretion experiments, during which time the rats were familiarized with Bollman-type restraining cages. After they had been deprived of food for 24 h (free access to water), the rats were placed in restraining cages and given a subcutaneous injection of 10 ml Ringer solution in the neck to prevent dehydration. The stomachs were then washed with 3×20 ml tepid 0.9% saline and drained for 1 h. Gastric juice was collected (free drainage) through the fistula in three 30-min fractions (basal secretion), followed by three 1-h fractions (gastrin-stimulated secretion). They received subcutaneous infusion of gastrin-17 (0.5 nmol kg^{-1} h^{-1}) by a peristaltic pump (pump rate, 1 ml h^{-1}) for 3 h through a needle inserted under the skin in the neck. A dose of 5 nmol kg^{-1} h^{-1} is thought to produce maximal acid response (Andersson *et al*., 1996). Acid was determined by titration with 0.02 M NaOH to pH 7.0 using an automatic titration assembly (Metrohm Herisau, Switzerland) and expressed as μmol H^+ h^{-1}. The second 1-h fraction after the start of administration of gastrin was used to determine the stimulated acid output.

Experimental design

Dose-response study YF476 and YM022, 0.3, 3, 30, 300 or 1000 μmol kg^{-1}, were given as a bolus dose to 160 freely fed rats by gastric gavage or by subcutaneous injection. The rats (still freely fed) were killed 24 h after dosage (between 1000 and 1200 h). The oxyntic mucosal HDC activity and serum gastrin concentration in each rat were measured. Also the serum concentrations of YF476 were determined in those rats that received the drug by subcutaneous injection.

Time-course study A total of 278 rats received a single dose of 300 μmol kg^{-1} of YF476 or YM022 by either oral administration or subcutaneous injection. Rats were killed immediately before or 1, 2, 3, 4, 5, 7 and 9 days and 2, 4, 8 and 16 weeks after the injections. Rats that received the drug orally were killed immediately before or 1, 2, 4, 6 and 12 h and 1, 2, 3, 4, 5, and 7 days after treatment. All rats were killed between 1000 and 1200 h. The time of oral administration was adjusted to the time of sacrifice. Serum was collected from the rats that received YF476 by subcutaneous injection and the serum concentrations of UF476 were determined.

Inhibition of gastrin-stimulated acid secretion

In the study of acid secretion, gastric fistula rats received a single subcutaneous dose of YF476 (300 μmol kg^{-1}) or vehicle. The acid output was monitored before (basal secretion for 1–2 h) and 1, 2 and 3 h after the start of the subcutaneous infusion of gastrin. The acid response was measured before and 1, 2 and 4 weeks after the administration of YF476.

Determination of serum gastrin

Serum gastrin was measured by radioimmunoassay using gastrin antiserum 2604 (a kind gift from Professor J.F. Rehfeld) (Stadil & Rehfeld, 1973; Håkanson *et al*., 1974). Rat gastrin-17 was used as standard, and the serum gastrin concentration was expressed as pmol l^{-1}.

Determination of histidine decarboxylase

The oxyntic mucosa was homogenized in ice-cold 0.01 M sodium phosphate buffer, pH 7.4, to a concentration of a 100 mg wet weight/ml. Aliquots (80 μl) of the oxyntic mucosal homogenates were incubated with L-[1-^{14}C]-histidine (sp.act. 50 mCi $mmol^{-1}$.), 0.5 mM L-histidine, and 0.01 mM pyridoxal-5-phosphate in a total volume of 160 μl at 37°C for 1 h as described previously (Larsson *et al*., 1986). The HDC activity was expressed as pmol $^{14}CO_2$ mg^{-1} h^{-1}.

Determination of YF476 in serum

Each aliquot (500 μl) of serum was passed through a 3 ml MP3 plus (SPEC) solid phase extraction column. Prior to use, the column had been activated by the passage of 1 ml methanol followed by 1 ml MilliQ water. The sample was passed through the column with a rate of 1 ml min^{-1}. The column was washed with 2 ml 50 mM sodium acetate, pH 4.1, followed by 2 ml MilliQ water and 2 ml methanol. YF476 was eluted with 1 ml 1% ammonia in ethyl acetate. The eluate was evaporated under vacuum. The extraction recovery of YF476 was 75%. Hydroxynefazodon was used as internal standard. The residue was dissolved in 100 μl mobile phase (see below) and 40 μl was taken for analysis by HPLC. Conditions: column TSK gel-OD5 80 TS (4.6 mm × 150 mm); column temperature 35°C; mobile phase 50 mM KH_2PO_4: acetonitrile: methanol (55 : 40 : 9, v v^{-1}); flow rate 1 ml min^{-1}; detection UV 238 nm. The samples were read against standard solutions of YF476 in methanol. Serum calibration curve: Concentra-

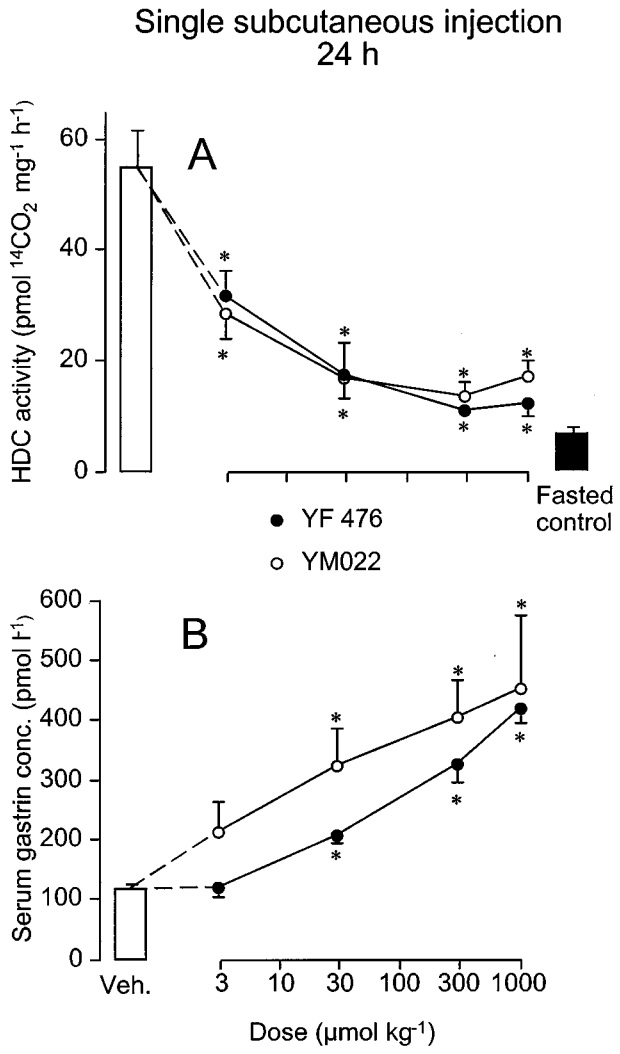

Figure 1 Dose-dependent effect of a single subcutaneous injection of YF476 and YM022 on the oxyntic mucosal HDC activity (A) and the serum gastrin concentration (B) in freely fed rats. The rats were killed 24 h after dosage. The HDC activity of fasted rats is given for comparison. Mean ± s.e.mean ($n = 7-12$). *$P < 0.01$ compared to vehicle.

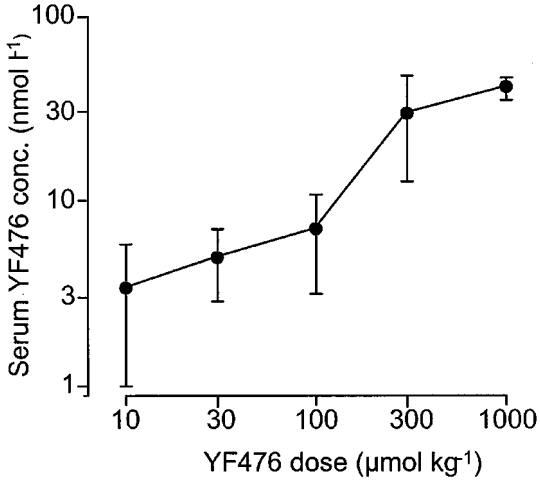

Figure 2 Serum concentration of YF476 (y-axis, logarithmic scale) 24 h after subcutaneous deposition of the drug. Different amounts of YF476 (x-axis, logarithmic scale) were given by a single bolus injection. Mean ± s.e.mean ($n = 4-8$).

tions in the range of 5 – 1000 nmol l^{-1} were prepared daily by adding 50 μl of calibrator marking solutions to 450 μl blank rat serum. The detection limit of the assay was 2 nmol l^{-1} (signal : noise, 3 : 1). The assay showed good linearity for YF476 in the range 5 – 1000 nmol l^{-1} with an assay performance of 3.9% (CV) within series and 15% (CV) between series at 11 nmol l^{-1}.

Statistical analysis

Results were expressed as means ± s.e.mean. Statistical significance was determined by one-way analysis of variance or Student's *t*-test. $P < 0.05$ was considered significant.

Results

Subcutaneous injection

YF476 and YM022, deposited subcutaneously as a single bolus injection, lowered the oxyntic mucosal HDC activity (Figure

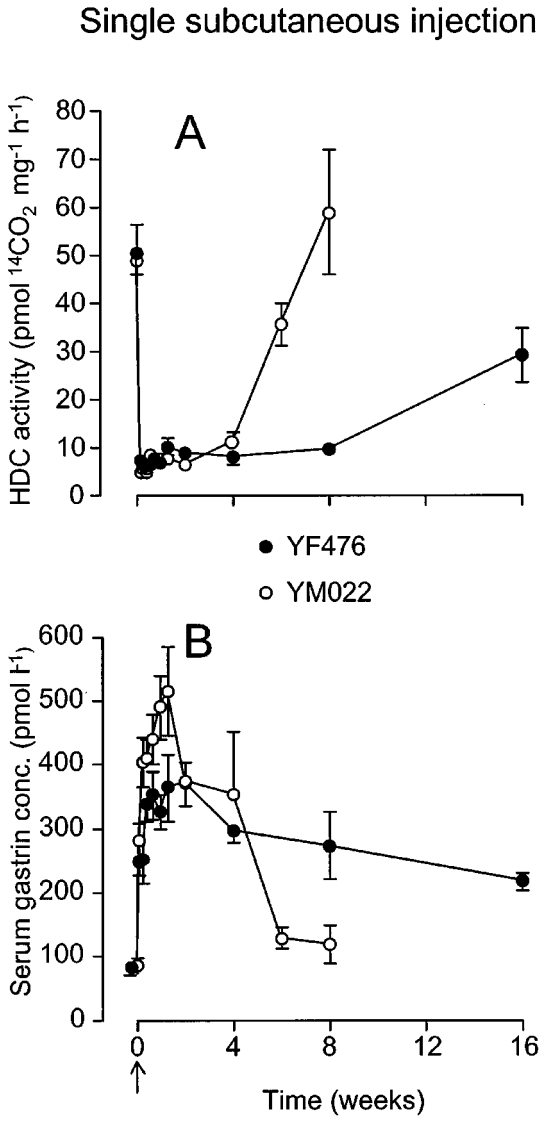

Figure 3 Long-lasting effect of a single subcutaneous injection of 300 μmol kg^{-1} of either YF476 and YM022 (at arrow) on the oxyntic mucosal HDC activity (A) and serum gastrin concentration (B). The rats were killed at different times after dosage. The values at zero time were obtained from untreated rats killed at the time of dosage. Mean ± s.e.mean ($n = 5-10$).

1A) and raised the serum gastrin concentration (Figure 1B) in a dose-dependent manner (measured 24 h after dosage) (freely fed rats). Maximum enzyme inhibition was achieved at a dose of 300 μmol kg^{-1} for both YF476 and YM022. This dose of YF476 resulted in a serum concentration of YF476 of 20–40 nmol l^{-1} (Figure 2) and lowered the HDC activity to the level seen in food-deprived rats, i.e. 10–20% of the level in freely fed rats (Figure 1A). The inhibition of HDC lasted for 4 (YM022) and 8–16 weeks (YF476), respectively (Figure 3A), and hypergastrinemia was observed in both groups for the same periods of time (Figure 3B). At sacrifice, drug residues could be seen at the injection site for as long as 4 (YM022) or 16 (YF476) weeks after injection, and measurable amounts of YF476 remained in the circulation for 4–8 weeks after the injection (Figure 4). A single subcutaneous injection of 300 μmol kg^{-1} of YF476 to fasted gastric fistula rats abolished the acid response to exogenous gastrin, while being without effect on the basal acid output (Figure 5A). The acid response to exogenous gastrin was abolished for at least 4 weeks (Figure 5B). The body weight development was not affected by either YM022 or YF476 (Figure 6).

Oral administration

YF476 and YM022, given by a single oral dose, lowered the oxyntic mucosal HDC activity in a dose-dependent manner (measured 24 h after dosage) (Figure 7A). YF476 raised the serum gastrin concentration (Figure 7B) and reduced the HDC activity to the level seen in fasted rats (Figure 7A). YM022 was less effective than YF476 in lowering the oxyntic mucosal

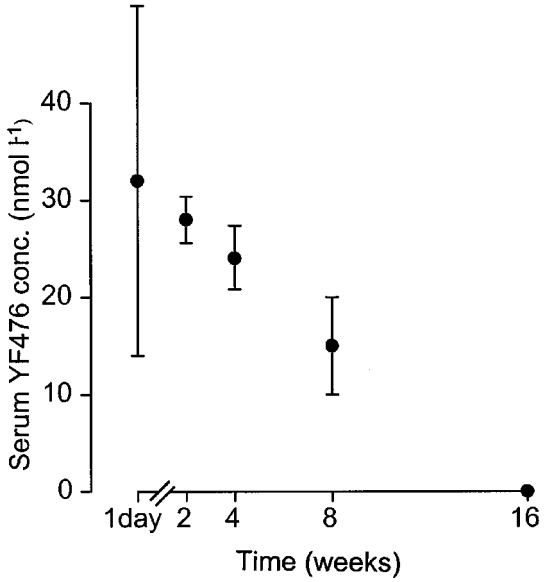

Figure 4 Serum concentration of YF476 (y-axis) (at sacrifice) after the subcutaneous deposition of 300 μmol kg^{-1} of YF476. The rats were killed at various times (x-axis) after dosage. Mean±s.e.mean ($n=5-8$).

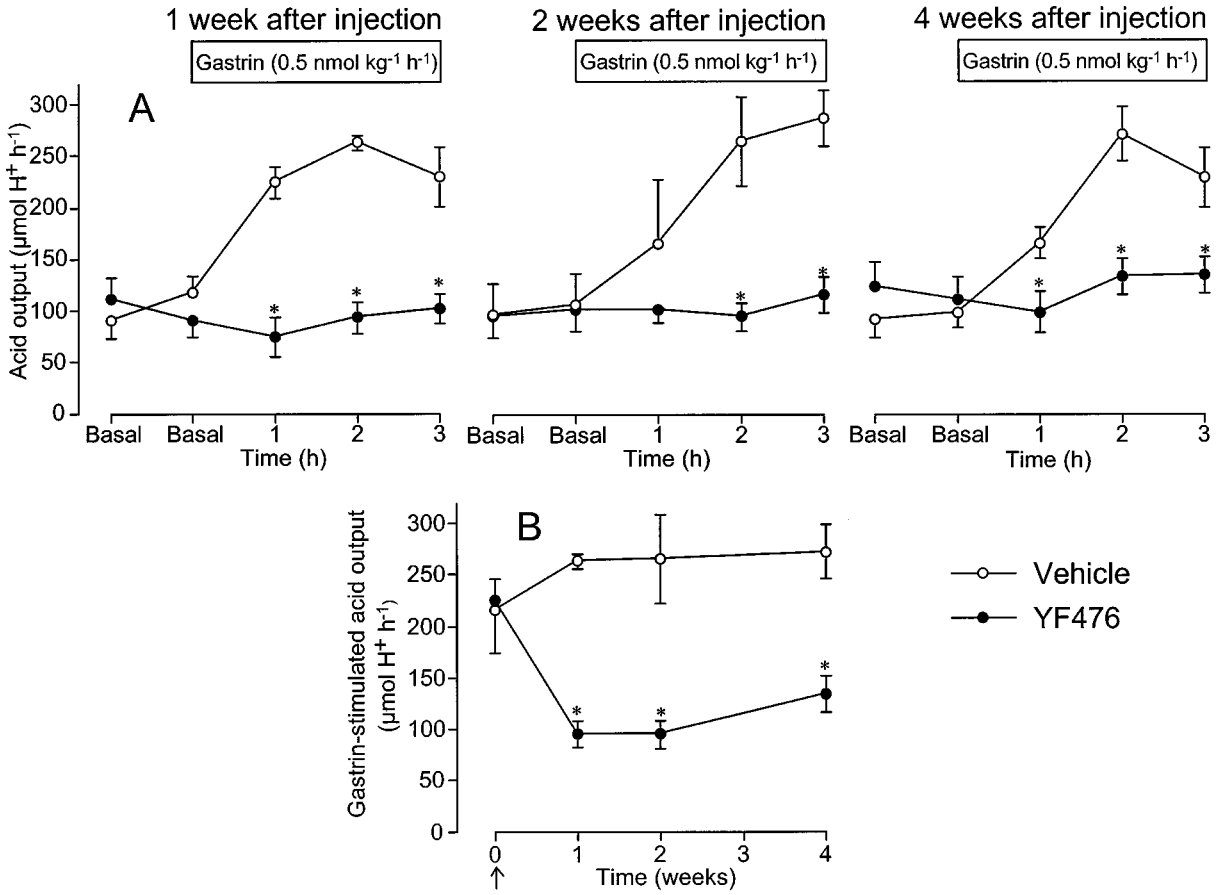

Figure 5 Gastrin-stimulated acid secretion (chronic gastric fistula rats) after a single subcutaneous injection of YF476 (3900 μmol kg^{-1}) or vehicle. Gastrin-17 (0.5 nmol kg^{-1} h^{-1}) was given by subcutaneous infusion for 3 h (as indicated). Gastric juice was collected in 1-h fractions. Experiments were carried out 1, 2 or 4 weeks after dosage (A). The results are summarized in (B), showing the long effect duration of a single injection of YF476 on gastrin-stimulated acid secretion. The acid output refers to the second hour of gastrin infusion. Mean±s.e.mean ($n=4-8$). *$P<0.01$ compared to vehicle.

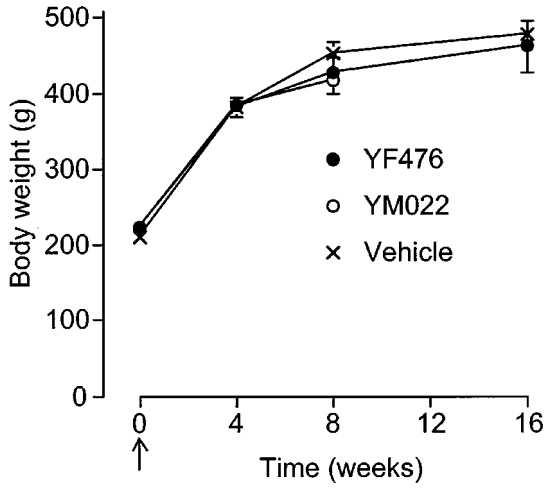

Figure 6 Body weight gain during the time of study in response to the subcutaneous deposition of 300 μmol kg^{-1} of YF476 and YM022 (at arrow). Vehicle was given to age matched control rats. Mean$\pm$s.e.mean ($n=6$).

HDC activity and failed to raise the serum gastrin concentration. The time-course of effect of a single oral dose of YF476 or YM022 (300 μmol kg^{-1}) on the HDC activity and serum gastrin concentration is shown in Figure 8. With both drugs, the reduction in HDC activity was prompt and lasted for 1 day in the case of YM022 and for 2–4 days in the case of YF476. The increase in serum gastrin concentration induced by YF476 reached maximum after 6–12 h and persisted for about 3 days.

Discussion

The gastrin-evoked activation of the ECL cells, manifested in the mobilization of histamine and activation of HDC, can be prevented by CCK$_2$ receptor blockade. This has been well documented in experiments with isolated ECL cells (Prinz *et al*., 1993; Lindström *et al*., 1997; 1999), vascularly perfused rat stomach preparations (Sandvik & Waldum, 1991) and intact rats (Ding *et al*., 1995; Ding & Håkanson, 1996a). Sustained CCK$_2$ receptor blockade lowers the HDC activity of the ECL

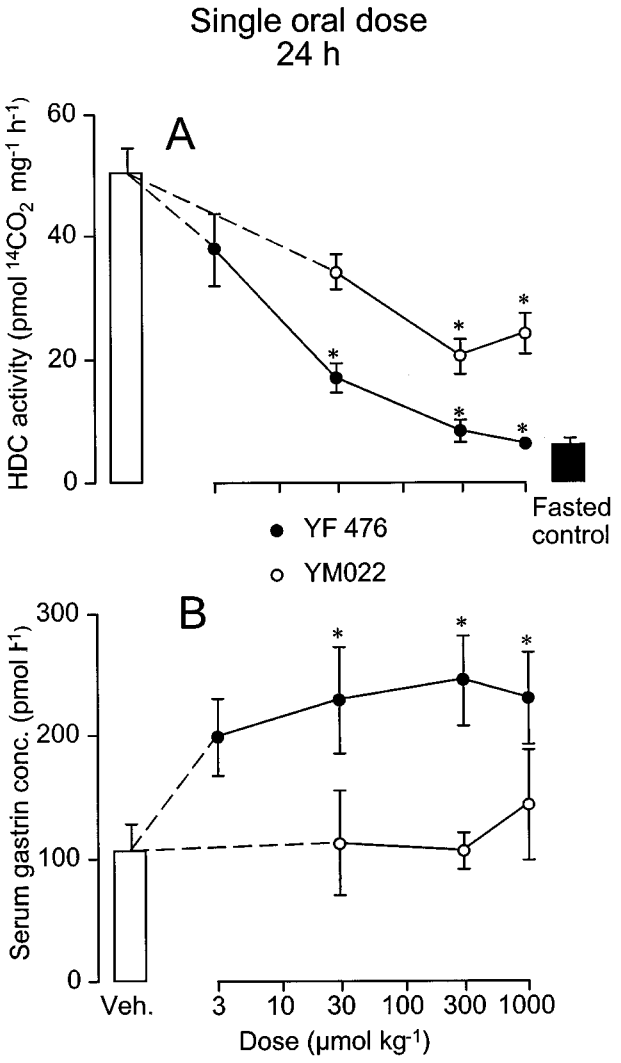

Figure 7 Dose-dependent effect of a single oral dose of YF476 or YM022 on the oxyntic mucosal HDC activity (A) and serum gastrin concentration (B) in freely fed rats at the time of sacrifice 24 h after dosage. The HDC activity of fasted rats is given for comparison. Mean$\pm$s.e.mean ($n=7-12$). *$P<0.01$ compared to vehicle.

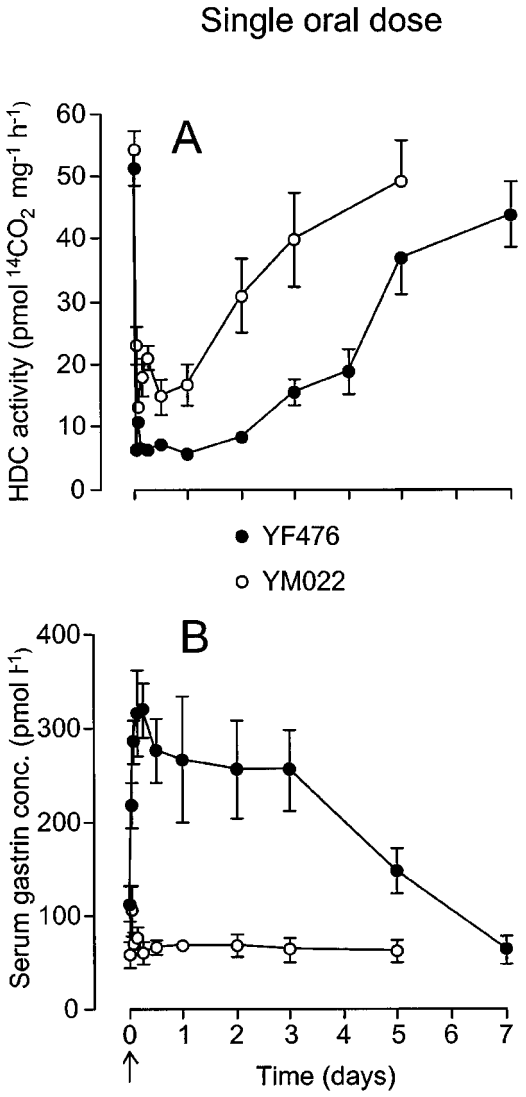

Figure 8 Long-lasting effect of a single oral dose of 300 μmol kg^{-1} of either YF476 or YM022 (at arrow) on the oxyntic mucosal HDC activity (A) and serum gastrin concentration (B). The rats were killed at different times after dosage. The values at zero time were obtained from untreated rats. Mean$\pm$s.e.mean ($n=5-10$).

cells and prevents them from responding to gastrin (Ding & Håkanson, 1996a,b; Ding *et al*., 1997a; Chen *et al*., 2000). The consequences of CCK_2 receptor blockade in intact freely fed rats include not only a lowered oxyntic mucosal HDC activity (Ding *et al*., 1997a,b) and acid blockade (Ding & Håkanson, 1996b) but also hypergastrinemia (Ding *et al*., 1997a,b), probably reflecting a reduced gastric acid output because of impaired secretion of histamine from the ECL cells. The present report describes the effects in rats of different bolus doses of YM022 or YF476, given by either the oral or the subcutaneous routes. The primary parameter measured was the oxyntic mucosal HDC activity, but also the serum gastrin concentration and the acid response to gastrin (in the case of subcutaneous YF476) were monitored. The body weight gain was not affected by either drug. Hence, neither long-term hypergastrinemia (Larsson *et al*., 1986) nor CCK_2 receptor blockade seem to interfere with food intake.

Earlier, both YM022 and YF476, given by continuous intravenous infusion, have been found to antagonize gastrin-evoked HDC activation in fasted rats with ID_{50} values of 2 nmol kg^{-1} h^{-1} (YF476) and 4 nmol kg^{-1} h^{-1} (YM022), and both drugs have been found to prevent also the omeprazole-evoked HDC activation (Ding *et al*., 1997a,c; Chen *et al*., 2000). The present study shows that a single dose of 300 μmol kg^{-1} of either YM022 or YF476, deposited subcutaneously, exerts remarkably long-lasting effects on the ECL cells, manifested in reduced HDC activity and increased serum gastrin concentration. After a single subcutaneous injection of YF476 to gastric fistula rats, an ED_{50} dose of gastrin failed to stimulate acid secretion for at least 4 weeks. This is consistent with the long-term suppression of HDC activity and elevation of serum gastrin concentration noted in these animals for as long as 8 weeks after the injection. There is compelling evidence that a single subcutaneous injection of maximally effective doses of either YM022 or YF476 generates deposits from which the drug is being absorbed slowly and over a long period of time: (a) the relative ability of YF476 to enter the blood stream appears to be reduced by increasing the drug load as evidenced by the apparent saturation effect of high doses on the circulating concentration, (b) the long duration of the HDC inhibition (with YF476 in particular), (c) the persistent presence of YF476 in the circulation, and (d) the demonstration of visible residues of YM022 and YF476 at the injection site weeks and even months after the injection. Hence, the long effect duration of YF476 and YM022 can be explained best by slow absorption of the drug from a subcutaneous deposit. It is unlikely that it reflects an irreversible or unsurmountable blockade of the CCK_2 receptors since stopping the continuous subcutaneous infusion of YM022 after 4 weeks led to the prompt reversal of the CCK_2 receptor blockade (Norlén *et al*., 1999). The measured serum concentration of YF476 ranged from 20–40 nmol l^{-1} 1 day after administration to about 15 nmol l^{-1} 8 weeks after administration. This concentration interval agrees well with the YF476 concentration needed to inhibit histamine secretion from isolated ECL cells (Lindström *et al*., 1999). From the latter report it is obvious that 20–40 nM YF476 is capable of causing near-maximal inhibition of the CCK_2 receptors. The pIC_{50} value in these experiments was calculated to be around 2 nM (calculated from data obtained from studies conducted in the presence of 10 nM gastrin), which agrees fairly well with the circulating concentrations needed in the present study to induce half-maximal inhibition of oxyntic mucosal HDC.

Oral administration of YF476 induced CCK_2-receptor blockade for a few days only. The effect was manifested in reduced HDC activity and increased serum gastrin concentration. In contrast, oral YM022 (at the doses tested) lowered the HDC activity partly and transiently but did not raise the serum gastrin concentration. The failure of oral YM022 to raise serum gastrin may be explained as follows: The serum gastrin concentration is known to increase whenever the gastric acid secretion is inhibited, but it probably needs to be inhibited significantly before there is a rise in serum gastrin. Apparently, oral YM022 does not inhibit acid secretion to the extent that serum gastrin is affected. Gastric acid secretion is controlled by the secretion of histamine from the ECL cells. If the secretory activity of the ECL cells is suppressed, the acid output is reduced accordingly. However, HDC has to be inhibited significantly before there is an effect on the amounts of histamine that can be mobilized (Norlén *et al*., to be published). Oral YM022 did not induce more than 60% inhibition of HDC, which probably does not have much effect on the amounts of histamine that can be mobilized from the ECL cells.

In summary, a single, maximally effective dose of 300 μmol kg^{-1} of YF476 or YM022, given by the subcutaneous route, suppressed the ECL cell activity for at least 4 (YM022) or 8 weeks (YF476) as manifested in greatly reduced HDC activity, greatly elevated serum gastrin level and (demonstrated in the case of YF476) abolished gastrin-stimulated acid secretion. The same dose of the two drugs, given by the oral route, induced 1 (YM022) or 2 (YF476) days suppression of ECL cell activity. Thus, (1) the effects of YF476 were more long-lasting than those of YM022 regardless of the mode of administration, and (2) subcutaneous deposition of either drug produced more long-lasting effects than did oral administration. In view of its high potency and long duration of action YF476 seems to be the drug of choice and deposition of the drug by a single subcutaneous injection seems to be the preferred mode of administration.

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