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Structural requirements for roxatidine in the stimulant effect of rat gastric mucin synthesis and the participation of nitric oxide in this mechanism

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1 The structural requirements of the histamine H_2-receptor antagonist, roxatidine (2-acetoxy-*N*-(3-[*m*-(1-piperidinylmethyl)phenoxy]-propyl)acetamide hydrochloride), for the stimulant effect on mucin biosynthesis and their relation to histamine H_2-receptor antagonism were identified by considering the structural analogues of this drug using an organ culture system of the rat stomach and competition studies with [^{125}I]iodoaminopotentidine ([^{125}I]-APT) binding to membranes of the guinea pig striatum.

2 [^{3}H]Glucosamine incorporation into mucin during 5 h incubation period was stimulated by roxatidine and its structural analogues A (2-hydroxy-*N*-(3-[*m*-(1-piperidinylmethyl)phenoxy]-propyl)acetamide) and B (*N*-(3-[*m*-(1-piperidinylmethyl)phenoxy]-propyl)acetamide). This effect was seen in mucosal cultures of the corpus, but not antrum, region.

3 Structural analogues, in which the length of the flexible chain between the benzene ring and the amide structure differs from that of roxatidine, failed to activate mucin synthesis. No significant change in mucus synthesis occurred with the addition of analogues in which the piperidine ring attached to the benzene ring via a methylene bridge was changed.

4 Specific [^{125}I]-APT binding to the histamine H_2 receptor of guinea pig brain membranes was inhibited by roxatidine and all structural analogues used in this study, except F (*N*-(3-[*m*-(*N*, *N*-dimethyl-aminomethyl)phenoxy]-propyl)acetamide).

5 Ranitidine at 10^{-4} M did not suppress the roxatidine-induced increase in [^{3}H]glucosamine incorporation into mucin.

6 Roxatidine-induced stimulation of [^{3}H]glucosamine incorporation into mucin was completely blocked by the addition of either N^G-nitro-L-arginine (10^{-5} M) or 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide sodium salt (10^{-5} M). The inhibitory action of N^G-nitro-L-arginine was totally reversed by L-arginine (5×10^{-3} M).

7 These results suggest that the cardinal chemical features of roxatidine for the activation of mucin biosynthesis in the corpus region of the rat stomach are the appropriate length of the flexible chain between the amide structure and the aromatic ring system bearing the methylpiperidinyl group at the meta position. The activity of roxatidine and its analogues to stimulate mucin synthesis is not related to their histamine H_2 receptor antagonistic activity. Roxatidine-induced activation of mucin biosynthesis in the corpus tissue is mediated by nitric oxide.

Keywords: Roxatidine; histamine H_2-receptor antagonists; gastric mucin biosynthesis; nitric oxide (NO); organ culture

Introduction

The clinical pharmacology of histamine H_2-receptor antagonists and their usefulness in the treatment and prevention of acid-peptide disorders have been reviewed (Feldman & Burton, 1990a,b). Some of the histamine H_2-receptor antagonists have been reported not only to inhibit acid secretion but also to promote gastric mucosal protection and the so-called process of cytoprotection (Shiratsuchi *et al*., 1988; Okabe *et al*., 1992; Ichikawa *et al*., 1994a). Compared with the structural requirements and acid-inhibitory mechanisms for the histamine H_2-receptor antagonists (Ganellin *et al*., 1976; Durant *et al*., 1978; Holtje & Batzenschlager, 1990), the chemical aspects for cytoprotective/gastroprotective actions have not been well defined because of the complicated mechanisms of mucosal protection. Previously, we reported that some acid inhibitory anti-ulcer drugs, which have cyto/gastroprotective actions, stimulated mucin biosynthesis in the corpus region of the rat stomach, indicating that the stimulation of mucin synthesis was closely related to mucosal protective activity (Ichikawa *et al*., 1994b). Furthermore, we showed that certain chemical properties of the amide structure and a six-membered aromatic ring, such as benzene and pyridine derivatives, were responsible for the activation of mucin biosynthesis by histamine H_2-receptor antagonists with cytoprotective actions (Ichikawa *et al*., 1996).

Roxatidine (2-acetoxy-*N*-(3-[*m*-(1-piperidinylmethyl)phenoxy]-propyl)acetamide hydrochloride), a second-generation histamine H_2-receptor antagonist (Tarutani *et al*., 1985) with an increased action on gastric mucosal defense (Shiratsuchi *et al*., 1988; Okabe *et al*., 1992), contains both a six-membered aromatic ring and an amide structure (Figure 1) and has a stimulant effect on corpus mucin biosynthesis in rat gastric mucosa (Ichikawa *et al*., 1994b). In the first step of the present study, we examined the structural requirements of this drug for the stimulant effect on rat gastric mucin biosynthesis, particularly with regard to whether the cardinal features of roxatidine are only the six-membered aromatic ring and amide structure, and its relation to histamine H_2-receptor antagonism. For this purpose, the biosynthesis of mucin in response to roxatidine and six related compounds was examined using an organ culture system of the rat stomach, and the histamine H_2-

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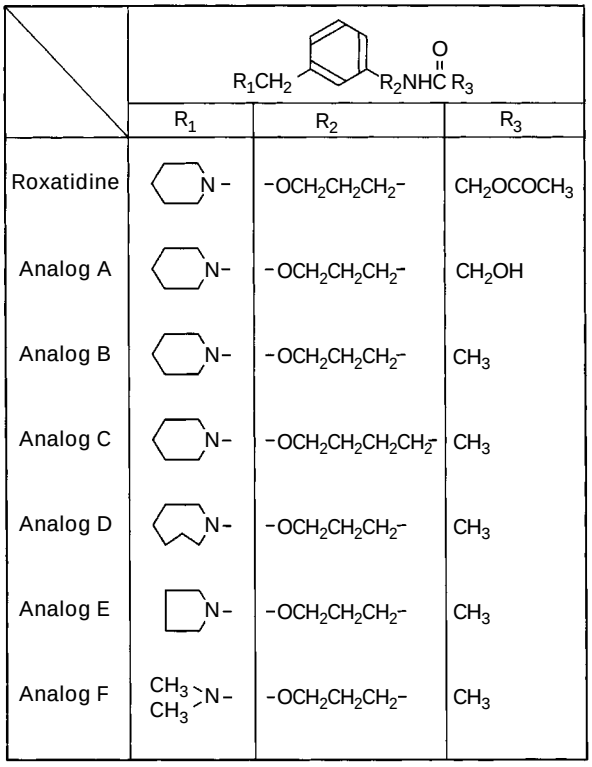

Figure 1 Chemical structures of roxatidine and its analogues used in this study.

receptor antagonistic properties of these compounds were investigated by competition studies with [^{125}I]iodoaminopotentidine ([^{125}I]-APT) binding to membranes of the guinea pig striatum. The six compounds bearing a benzene ring and an amide structure like roxatidine are 2-hydroxy-*N*-(3-[*m*-(1-piperidinylmethyl)phenoxy]-propyl)acetamide, analogue A; *N*-(3-[*m*-(1-piperidinylmethyl)phenoxy]-propyl)acetamide, analogue B; *N*-(4-[*m*-(1-piperidinylmethyl)phenoxy]-butyl)acetamide, analogue C; *N*-(3-[*m*-(1-azacycloheptylmethyl)phenoxy]-propyl)acetamide, analogue D; *N*-(3-[*m*-(1-pyrrolidinylmethyl)phenoxy]-propyl)acetamide, analogue E and *N*-(3-[*m*-(*N*,*N*-dimethylaminomethyl)phenoxy]-propyl)acetamide, analogue F (Figure 1). The second aim of this study was to clarify whether endogenous nitric oxide (NO), known as a labile free radical and suggested as a mediator in an increasing variety of physiological processes in many tissues (Whittle, 1994), contributes to roxatidine-induced stimulation of gastric mucin biosynthesis in rat gastric mucosa using a new class of NO antidote, a direct scavenger of NO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide sodium salt (carboxy-PTIO) (Yoshida *et al*., 1994).

Methods

Experimental animals

Seven-week-old male Wistar rats (SLC, Shizuoka, Japan) each weighing approximately 170 g were used. All were fasted for 24 h before the experiments and had free access to water during this time.

Organ culture

The stomachs were excised immediately after the rats had been euthanized with CO_2. The stomachs were then cut along the greater curvature, and the luminal surface was gently washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS(−)). The glandular part was selected, separated into the corpus and antrum, and cut into small 2×2 mm sections. The tissue culture method of Eastwood & Trier (1973) was used with a modification (Ichikawa *et al*., 1993). Six tissue fragments were randomly picked from six different stomachs and were placed, with the mucosal surface facing up, on a stainless steel grid in the central wall of a plastic culture dish (60×15 mm, Falcon, U.S.A.) containing 0.75 ml of the culture medium and 0.05 ml of drugs. The medium consisted of 90% Eagle's minimum essential medium and 10% dialysed foetal calf serum, with 370 kBq ml^{-1} of D-[1,6-^{3}H(*N*)]-glucosamine hydrochloride (1943 GBq mmol^{-1}, New England Nuclear). All dishes were maintained at 37°C in 5% CO_2 and 95% air. Incubation period was fixed to 5 h throughout this study.

Isolation of labelled mucin and radioactivity measurements

Upon completion of the culture period, the tissue fragments were harvested from the medium, gently rinsed with PBS(−) and boiled at 100°C for 3 min in 0.4 ml of 0.05 M Tris-HCl buffer, pH 7.2. The extraction and isolation of mucin were performed as previously described (Ichikawa *et al*., 1993). The tissue fragments were homogenized using a Physcotron microhomogenizer (Niti-On, Chiba, Japan). Triton X-100 was added to a 2% (v/v) concentration, and the homogenate was shaken for 1 h at 37°C. The homogenate thus obtained was centrifuged at 8000 *g* for 30 min. A 0.4 ml portion of the supernatant was applied onto a Bio-Gel A-1.5 m column (1×30 cm) previously equilibrated with Tris buffer containing 2% Triton X-100, and the column was eluted with this buffer. Finally, fractions of 0.8 ml each were collected, and the radioactivity was measured using a scintillation counter (Beckman, Model LS-2800, U.S.A.) with Aquasol-2 (New England Nuclear, U.S.A.) as the scintillant. The radioactivity recovered into the void volume fractions of the column, which had been demonstrated to be the synthesized mucin (Ichikawa *et al*., 1993), was determined. To compare mucin synthesis rates, the total radioactivity of these fractions was divided by the tissue protein content of each homogenate to give the value relative to that of the control.

Histamine H_2-receptor binding

According to a method previously described (Ruat *et al*., 1990; Leurs *et al*., 1994), the histamine H_2-receptor antagonistic properties of roxatidine and its structural analogues were investigated by competition with the binding of the rabiolabelled H_2-receptor antagonist [^{125}I]-APT to membranes of the guinea pig striatum by CEREP (Celle l'Evescault, France). The preparation of brain membranes from male Hartley guinea pigs and the method used for [^{125}I]-APT labelling have been previously described in detail (Raut *et al*., 1990). For competition experiments, 0.1 nM [^{125}I]-APT was incubated with 70 mg of membrane proteins in the presence of varying concentrations of test compounds (roxatidine and ranitidine: 1×10^{-8} to 3×10^{-6} M, six concentrations; analogues A ~ F: 3×10^{-8} to 1×10^{-5} M, six concentrations) for 150 min at 22°C. Following incubation, the membranes were rapidly filtered under vacuum through GF/B glass fibre filters (Packard). The filters were then washed several times with an ice-cold buffer using a Packard cell harvester. Bound radioactivity was measured with a scintillation counter (Topcount, Packard) using a liquid scintillation cocktail (Microscint 0, Packard). The compounds were tested at six concentrations in duplicate to obtain competition curves. In the same experiment, the reference compound (cimetidine) was simultaneously tested at six concentrations in duplicate to obtain a competition curve in order to validate these experiments. The specific radioligand binding to the receptors is defined as the difference between total binding and non-specific binding determined in the presence of an excess of unlabelled ligand. Results are expressed as a percentage of control specific binding obtained in the presence of the test

compounds. IC_{50} values (concentration required to inhibit 50% of specific binding) and Hill coefficients (nH) were determined by nonlinear regression analysis of the competition curves. These parameters were obtained by Hill equation curve fitting.

Drugs

The following drugs were obtained for use in this study: Roxatidine and six analogues A ~ F, which bear a structural resemblance to roxatidine (Teikoku Hormone Mfg. Co., Ltd, Kawasaki, Japan); Ranitidine hydrochloride (Sigma, U.S.A.); N^G-nitro-L-arginine (L-NOARG) (Wako Pure Chemical Industries, Osaka, Japan); Carboxy-PTIO (Dojindo Laboratories, Kumamoto, Japan). Roxatidine and its analogues were dissolved in dimethyl sulfoxide (DMSO) and added at concentrations of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M to the dishes, making the final concentration of DMSO 0.01%. To assess the stability of these drugs, the concentrations of each test substance in the medium before and after 5 h incubation were determined by gas chromatography-mass spectrometry under the same conditions used in previous studies (Iwamura *et al*., 1985; Nagai *et al*., 1995). At least 90% of roxatidine and its analogues dissolved in the medium could be recovered as intact forms after 5 h incubation, indicating that all the drugs were similarly stable during contact with the tissue. Ranitidine, L-NOARG and carboxy-PTIO were dissolved in distilled water.

Protein determination

The protein content in the tissue homogenate was determined by the bicinchoninic acid method (Smith *et al*., 1985) with a Pierce protein assay kit, using bovine serum albumin as the standard.

Statistical analysis

The results were expressed as means ± s.d. The one-way analysis of variance (ANOVA) with Scheffe's test was used for statistical analysis with $P<0.05$ taken as significant.

Results

Influence of roxatidine and its structural analogues on mucin biosynthesis in the corpus region

Figure 2 shows the mucin biosynthetic activity of the corpus as measured by [^{3}H]glucosamine incorporation. The biosynthesis of mucin in the control (0.01% DMSO alone) was 12003 ± 1393 dpm mg^{-1} tissue protein. In the corpus, the addition of 10^{-6} M of roxatidine, the analogues A and B enhanced [^{3}H]glucosamine incorporation into the mucin, but mucin biosynthesis was not susceptible to the addition of the other analogues (Figure 2). The biosynthetic responses to analogues A and B were essentially the same as that to the roxatidine-treatment group (Figure 3).

Influence of roxatidine and its structural analogues on mucin biosynthesis in the antral region

In the antrum, no significant change could be detected in mucin biosynthesis with the addition of any drug ([^{3}H] specific activity: 25820 – 28567 dpm mg^{-1} tissue protein (data not shown)).

Affinity of roxatidine and its structural analogues for the histamine H_2-receptor

The effect of roxatidine and its structural analogues on [^{125}I]-APT binding to guinea pig brain membranes is shown in Figure 4. Specific [^{125}I]-APT binding (0.1 M) to guinea pig striatum preparations was inhibited in a monophasic manner and with similar potencies by roxatidine and ranitidine (Figure 4a and Table 1). Analogues A and B showed similar IC_{50} values and were more potent than roxatidine. The other com-

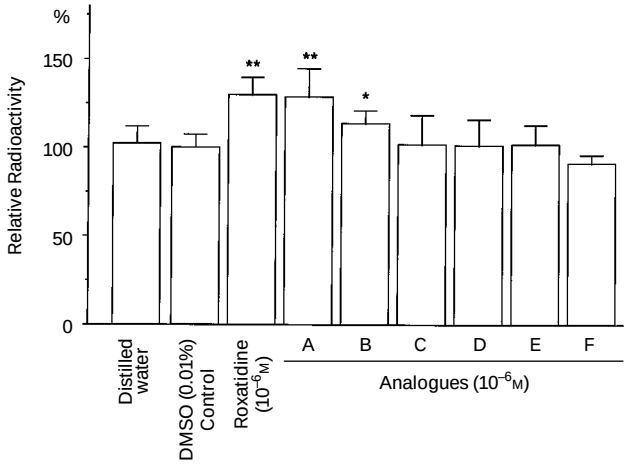

Figure 2 Influence of roxatidine (10^{-6} M) and its structural analogues (10^{-6} M) on [^{3}H]glucosamine incorporation into mucins in the corpus region. Values are expressed as percentages of 0.01% DMSO alone group (control) and represent means ± s.d. from six different samples derived from six different animals. $*P<0.05$, $**P<0.01$ compared to the control value.

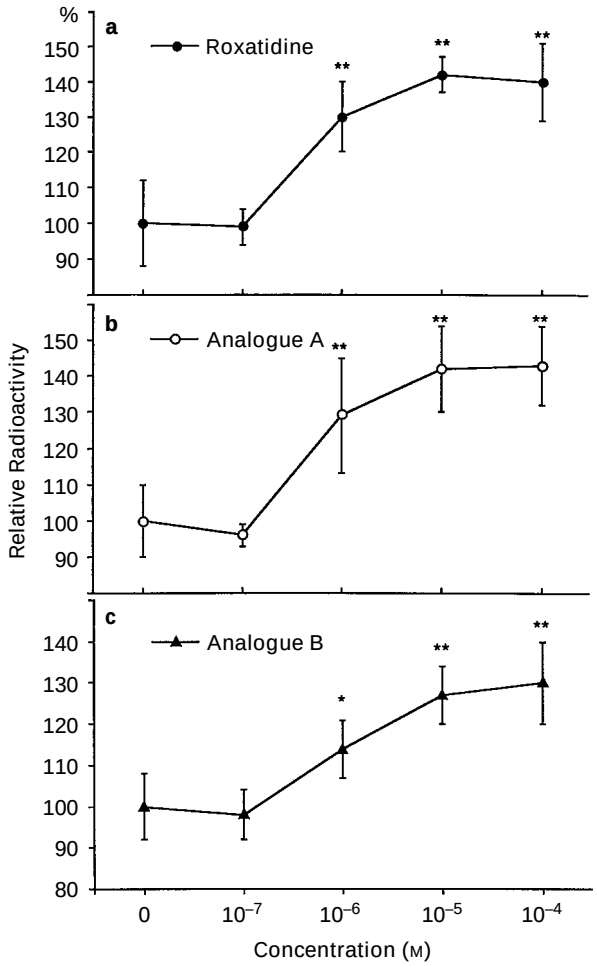

Figure 3 Effect of roxatidine (a), analogues A (b) and B (c) on [^{3}H]glucosamine incorporation into mucin in corpus tissue. Values are expressed as percentages of controls and represent means ± s.d. from six different samples derived from six different animals. $*P<0.05$, $**P<0.01$ compared to control value (0 M).

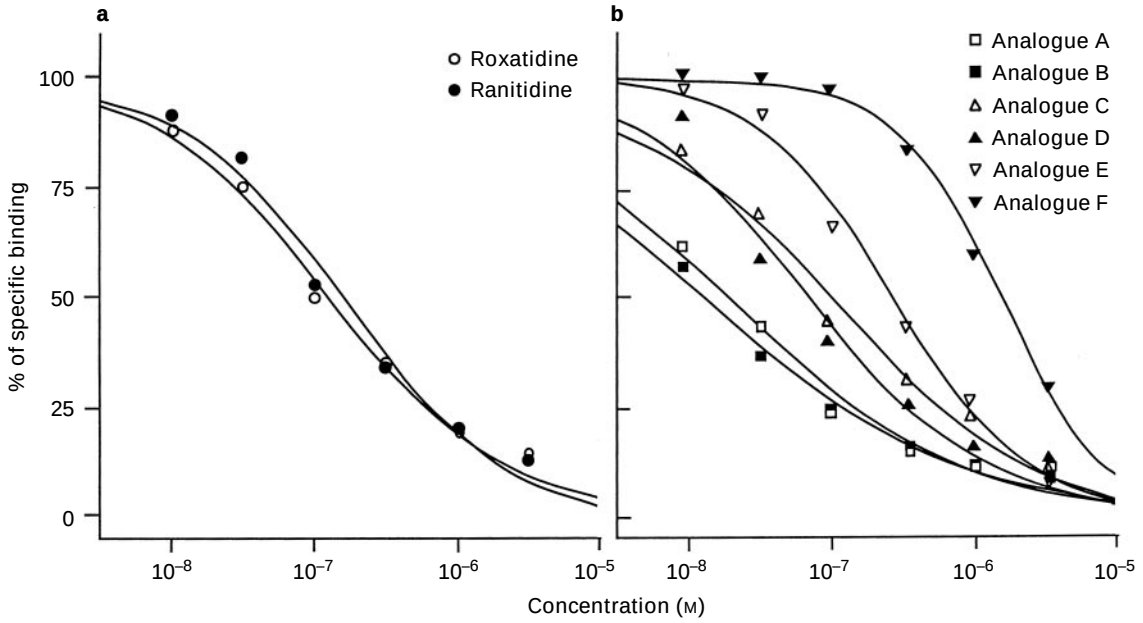

Figure 4 Pharmacological profile of [^{125}I]iodoaminopotentidine ([^{125}I]-APT) binding to membranes of guinea pig striatum. Displacement of [^{125}I]-APT binding by (a) roxatidine, ranitidine or (b) roxatidine structural analogues. Data shown are the mean values of duplicate determinations from a typical experiment.

Table 1 IC_{50} values of roxatidine and its structural analogues for competition with [^{125}I]iodoaminopotentidine binding to the histamine H_2 receptor in membranes of guinea pig striatum

Compounds	*IC_{50}* (nM)	*nH*
Roxatidine	128	0.71
Analogue A	62	0.54
Analogue B	43	0.50
Analogue C	369	0.62
Analogue D	220	0.72
Analogue E	782	0.93
Analogue F	4680	1.19
Cimetidine	588	0.64

Values are expressed as means of two determinations.

pounds, except analogue F, also displaced the specific [^{125}I]-APT binding to histamine H_2 receptors. In contrast, analogue F was poorly active (Figure 4b and Table 1).

Influence of the histamine H_2-receptor antagonist, ranitidine, on the roxatidine-induced increase in mucin synthesis

The 10^{-6} M roxatidine-induced increase of [^{3}H]glucosamine-labeled mucin in the corpus was not significantly suppressed by the addition of 10^{-4} M ranitidine (Heim *et al*., 1990; Figure 5).

Influence of the NO synthase inhibitor, L-NOARG, on the roxatidine-induced increase in mucin synthesis

Administration of L-NOARG (10^{-5} M) did not significantly change the biosynthesis of mucin in the corpus. L-NOARG completely suppressed the 10^{-6} M roxatidine-induced increase in [^{3}H]glucosamine-labeled mucin in the corpus at a concentration of 10^{-5} M (Figure 6). This inhibitory action of L-NOARG was totally reversed by L-arginine (5×10^{-3} M).

Influence of the NO scavenger, carboxy-PTIO, on the roxatidine-induced increase in mucin synthesis

No significant change could be detected in the mucin biosynthesis in the corpus with the addition of carboxy-PTIO

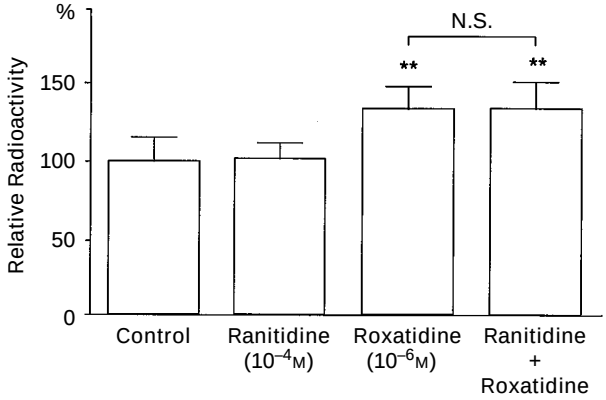

Figure 5 Influence of ranitidine (10^{-4} M) on the roxatidine (10^{-6} M)-stimulated incorporation of [^{3}H]glucosamine into mucin in corpus tissue. Values are expressed as percentage of controls and represent means ± s.d. from six different samples. Asterisks indicate statistical significance (**$P<0.01$) versus control value.

(10^{-5} M). The roxatidine-induced increase in [^{3}H]-labelled mucin in the corpus was completely blocked by addition of 10^{-5} M carboxy-PTIO (Figure 7).

Discussion

There is compelling evidence that the six-membered aromatic ring and the amide structure in the second-generation histamine H_2-receptor antagonists are essential for the stimulation of mucin production, which may be strongly correlated with the cytoprotective action of these drugs (Ichikawa *et al*., 1996). Six compounds containing both a benzene ring and an amide structure were used in the present work with structural analogues of roxatidine (Figure 1). Analogues A, B and C bear the piperidine ring (indicated by R1 in Figure 1) attached to the benzene ring via a methylene bridge, but the length of the flexible chain (indicated by R2 in Figure 1) of analogue C differs from that of roxatidine. This study shows that analogues A and B, but not C, stimulated corpus mucin biosynthesis in a manner similar to that of roxatidine. This means that the

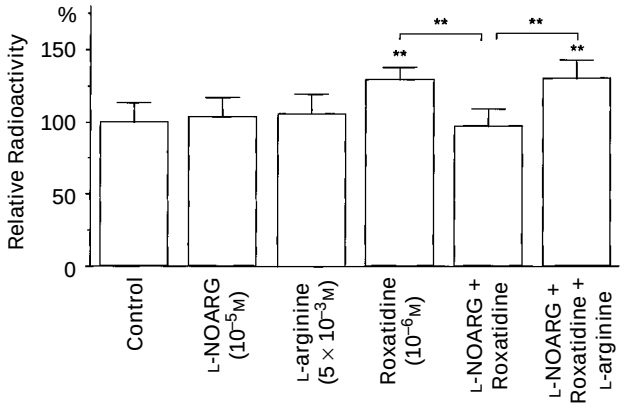

Figure 6 Influence of L-NOARG (10^{-5} M) on the roxatidine (10^{-6} M)-stimulated incorporation of [^{3}H]glucosamine into mucin in corpus tissue. Values are expressed as percentages of controls and represents means ± s.d. from six different samples. Asterisks indicate statistical significance (**$P<0.01$), those just above the s.d. bar showing the significance versus the control value.

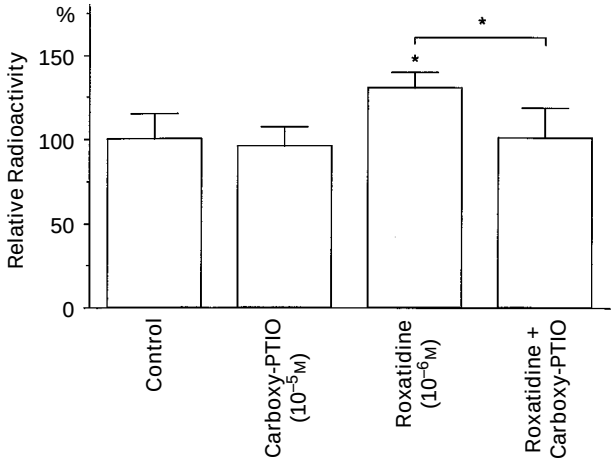

Figure 7 Influence of carboxy-PTIO (10^{-5} M) on the roxatidine (10^{-6} M)-stimulated incorporation of [^{3}H]glucosamine into mucin in corpus tissue. Values are expressed as percentages of controls and represents means ± s.d. from six different samples. Asterisks indicate statistical significance (*$P<0.05$), those just above the s.d. bar showing the significance versus the control value.

length of the flexible chain between the benzene ring and the amide structure is essential for this stimulation. Analogues D, E and F having different ring structures or no ring structure at R1 of the roxatidine molecule failed to activate mucin biosynthesis. Analogues D, E and F contain the same flexible chain as roxatidine. Thus the piperidine ring is also important for their activity. Our data indicate that the structural requirements for the stimulant effect of roxatidine on mucin biosynthesis are not only the six-membered aromatic ring and amide structure, but that the attachment of the piperidinomethyl group and the appropriate length of the flexible chain are also important for this function.

All drugs utilized in the present study failed to change the biosynthesis of antral mucin. Gastrin, known to be a gastrointestinal hormone, significantly accelerated mucin biosynthesis only in the oxyntic region, but yielded no significant change in the antral region of the rat stomach (Ichikawa *et al*., 1993). In previous studies, mucins obtained from the corpus and antrum of rat gastric mucosa were shown to differ in their subunit structures and the chemical composition of their carbohydrate moieties (Ohara *et al*., 1988; Goso & Hotta, 1989). These results and immunohistochemical observations (Ishihara *et al*., 1996) strongly indicate that there are different types of mucus-producing cells in the corpus and antrum of the gastric mucosa, which may have a distinct mechanism for the regulation of mucin biosynthesis.

With regard to their histamine H_2-receptor antagonistic properties, the six analogues were characterized by their ability to complete with [^{125}I]-APT binding to membranes of the guinea pig striatum. This radiolabel has successfully been used for the labelling of human H_2 receptors in brain tissue (Traiffort *et al*., 1992). Aminopotentidine (APT) and iodoaminopotentidine (I-APT) have been shown to behave as insurmountable antagonists of histamine responses in reducing histamine-induced acid secretion in the anaesthetized rat (Coruzzi *et al*., 1996). Results obtained in the present study showed that all compounds except analogue F, displaced the specific [^{125}I]-APT binding to histamine H_2-receptor sites. The relative potencies of these antagonists were: analogue B > A > roxatidine > D > C > E. Compared with the IC_{50} value for cimetidine obtained under similar experimental conditions, roxatidine, analogues A, B, C and D were 4.6, 9.5, 13.7, 1.6 and 2.7 times more potent than cimetidine, respectively. These results suggest that the histamine H_2 receptor antagonism was not directly correlated with the roxatidine-induced stimulation of mucin biosynthesis. In this study, the histamine H_2-receptor antagonist ranitidine (Daly *et al*., 1981) did not suppress the increase in [^{3}H]glucosamine incorporation induced by 10^{-6} M roxatidine. Our data indicate that the histamine H_2 receptor, for which roxatidine competes with ranitidine, cannot be held responsible for the roxatidine-induced stimulation of mucin biosynthesis. Although little is understood about the regulatory mechanism of mucin production, acid secretagogues are claimed to participate in increasing mucin synthesis in isolated pig and canine gastric mucosal cells (Heim *et al*., 1990; 1991; Scheiman *et al*., 1992). Histamine has been shown to stimulate the production of glycoprotein in isolated mucous cells through the histamine H_2 receptor, analogous to histamine receptors present on the gastric parietal cells (Heim *et al*., 1990; Scheiman *et al*., 1992). Although differences between species and experimental systems should be kept in mind, the present data indicate that the roxatidine effect on mucin synthesis results from a different mechanism of action than the analogues effect of histamine. Using compounds related to FRG-8813, another second-generation histamine H_2-receptor antagonist containing both a six-membered aromatic ring and an amide structure, we showed that the stimulation of mucin biosynthesis did not directly correlate with their histamine H_2-receptor antagonism (Ichikawa *et al*., 1996). It is still uncertain whether roxatidine and FRG-8813 have a direct effect on mucus-producing cells or an indirect effect through the action on other cells. In any case, roxatidine, FRG-8813 and their structural analogues might be very useful tools for the detection and further clarification of the regulatory mechanism of mucin synthesis in the oxyntic region of the gastric mucosa.

There has been increased awareness of the potential of NO to take part in a variety of physiological processes in many tissues including the gastrointestinal tract (Whittle, 1994). Brown *et al*. (1992; 1993) reported that NO donors increased the thickness of the mucus layer in the rat stomach and induced mucus secretion by gastric mucosal cells without evidence of cellular damage. In contrast, a recent study using a cell culture system of highly enriched mucus cells of rabbit gastric mucosa demonstrated that the roxatidine-induced increase in mucus synthesis and secretion was not significantly suppressed by the addition of a nitric oxide synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA) (Takahashi & Okabe, 1995). To clarify whether endogenous NO contributes to the roxatidine-induced increase in mucin synthesis in the entire gastric mucosa, we examined the susceptibility of the roxatidine effect to the NOS inhibitor, L-NOARG (Mulsch & Busse, 1990), and the NO scavenger carboxy-PTIO (Akaike *et al*., 1993; Yoshida *et al*., 1994). Unlike the results obtained with the primary culture model of isolated mucus cells (Ta-

kahashi & Okabe, 1995), ours obtained with a tissue culture system show that NO itself plays an important role in mediating the gastric mucin synthesis in the corpus tissue elicited by roxatidine. Although the species difference between rat and rabbit should be kept in mind, our results suggest that roxatidine has an indirect effect on mucus-producing cells through the action of NO. Our previous studies have shown that gastrin also accelerates mucin biosynthesis in the oxyntic region of rat gastric mucosa (Ichikawa *et al*., 1993), and this activation involves NO synthesis. Whether roxatidine promotes gastric mucin biosynthesis *in vivo* remains to be shown by studying gastric mucin accumulation and/or secretion following the oral administration of this drug to rats (Ichikawa *et al*., 1994a).

In summary, the present study demonstrates that the cardinal chemical features of roxatidine for the activation of mucin biosynthesis in the corpus region of the rat stomach are the appropriate length of the flexible chain between the amide structure and the aromatic ring system bearing the methylpiperidinyl group at the meta position. The activity of roxatidine and its analogues to stimulate mucin synthesis is not related to their histamine H_2-receptor antagonistic activity. Roxatidine-induced activation of mucin biosynthesis in the corpus tissue is mediated by NO.

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