Effects of H₂-Receptor Antagonists on ³H-Cimetidine Binding and Histamine-Stimulation of Cellular cAMP in Isolated Guinea Pig Gastric Glands

Akira TANAKA, Shuichiro NISHIHARA, Tadashi MISAWA and Hiroshi IBAYASHI

The Third Department of Internal Medicine, Faculty of Medicine, Kyushu University, 1-1, 3 chome, Maidashi, Higashi-ku, Fukuoka 812, Japan

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Abstract—³H-Cimetidine binding to plasma membranes of isolated guinea pig gastric glands was investigated, and the effects of five H₂-receptor antagonists on ³H-cimetidine binding and histamine stimulation of cellular cAMP were compared. Of the five cations tested, Cu⁺⁺ markedly increased specific ³H-cimetidine binding. ³H-Cimetidine had high affinity (Kₐ=0.41×10⁻⁶ M) and low affinity (Kₐ=12.8×10⁻⁶ M) binding sites. Cimetidine and etintidine were potent inhibitors of ³H-cimetidine binding, while famotidine, ranitidine and TZU-0460 were not. Histamine stimulation of cellular cAMP was competitively inhibited by H₂-receptor antagonists yielding pA₂ values of 6.41 for cimetidine, 6.82 for etintidine, 6.87 for ranitidine, 6.94 for TZU-0460 and 7.60 for famotidine. Because the Kₐ value (log Kₐ=−pA₂) of 0.39×10⁻⁶ M for cimetidine is close to the Kₐ value for the high affinity ³H-cimetidine binding site, it is presumed to represent a part of the H₂-receptor, and the relative potency of etintidine against cimetidine in inhibiting ³H-cimetidine binding is similar to that in inhibiting histamine stimulation of cellular cAMP. These results suggest that imidazole-derived H₂-receptor antagonists (cimetidine and etintidine) and non-imidazole H₂-receptor antagonists (famotidine, ranitidine and TZU-0460) compete with histamine at different sites on the H₂-receptor of the gastric glands.

Since burimamide, metiamide and cimetidine had been reported to inhibit gastric acid secretion, it has generally been concluded that histamine-stimulated acid secretion of the stomach is mediated by a specific class of receptors classified as H₂-receptors (1, 2); cAMP is regarded as the second messenger for these sites (3–6). With the development of new H₂-receptor antagonists, which differ considerably from cimetidine in chemical structure, the concept of the structural requirements for H₂-receptor antagonism has changed (7, 8). For further investigation of the H₂-receptor, the radioligand binding technique has been used for examination of brain or gastric tissues (9–13). To study parietal cell function at the cellular level, preparations of enriched parietal cells such as isolated gastric glands or isolated parietal cells are often used. In the present work, we compared the inhibitory effect of five H₂-receptor antagonists (cimetidine, etintidine, ranitidine, TZU-0460 and famotidine; for structures, see Fig. 1) on ³H-cimetidine binding and histamine-stimulation of cellular cAMP in isolated guinea pig gastric glands.

Materials and Methods

1) Preparation of isolated gastric glands

Male Hartley albino guinea pigs (300–500 g) were deprived of food for 24 hr and then decapitated. The fundic region of the stomach was removed and rinsed several times with ice-cold 0.9% NaCl. The fundic mucosa was separated from the muscularis and cut into small pieces using a razor blade. Minced tissue was rinsed three times in oxygenated Hanks' MEM solution (with 0.2% bovine serum albumin, 25 mM HEPES,
pH 7.4) and digested with 0.05% collagenase enzyme solution at 37°C. During digestion, the suspension was gently shaken, and gassed continuously with 100% O₂. After 20 min of digestion, the suspension was allowed to settle, and the sediment was reincubated with fresh Hanks' MEM solution containing 0.05% collagenase at 37°C. After 20 min of digestion, the suspension of isolated gastric glands was filtered through nylon mesh and rinsed three times with ice-cold Hanks' MEM solution. The average populations of parietal cells, zymogen cells and other cells in the isolated gastric gland were 59.1±2.5%, 26.8±1.9% and 13.2±1.4% (Mean±S.E., n=15), respectively, as determined on the fluorescence micrographs of the isolated gastric glands in the presence of acridine orange (10⁻⁴ M). As the exclusion rate of 0.5% trypan blue was over 95%, the viability of isolated gastric glands was good.

2) Preparation of the plasma membranes from isolated gastric glands
Using a Teflon-glass homogenizer, the isolated gastric glands were homogenized in 0.25 M sucrose (3 mM Tris-HCl, pH 7.4) and then centrifuged at 700 x g for 10 min. The supernatant was recentrifuged at 45,000 x g for 20 min. After resuspension and recentrifugation had been performed twice, the pellet was gently suspended in 10 mM Tris-HCl buffer (pH 7.4). These procedures were carried out at 4°C, and membranes were stored at -20°C.

3) Experimental design
i) Cellular cAMP: Isolated gastric glands were suspended in Hanks' MEM solution (0.5–1 mg protein/ml). After preincubation for 5 min, 1 ml samples of the suspension of isolated gastric glands were incubated with each secretagogue at 37°C for 10 min, and then incubation was halted by cooling the samples at 4°C. Immediately afterwards, the sample was centrifuged at 700 x g for 5 min. The sediments were homogenized by ultrasonic disintegration (20 kHz, 10 sec) and extracted with 0.1 N HCl for cAMP assay. The cAMP assay was carried out using a Yamasa cAMP radioimmunoassay kit. Protein content of the gastric glands was determined by the method of Lowry et al. (14).

ii) ³H-Cimetidine binding to plasma membranes: Membranes preparations (50–100 μg protein/tube) were incubated with ³H-cimetidine in 14 mM Tris-HCl buffer (pH 7.4) at 4°C in a final volume of 250 μl. To study the effect of the incubation temperature, the incubation was also performed at 22 and 37°C.
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(pH 7.4). At the end of the incubation, 3 ml ice-cold buffer was added, and immediately afterwards, membranes were harvested by filtration under vacuum through Whatman GF/C filters. After the filters had been rinsed twice with Tris-HCl buffer, they were put into counting vials containing the counting solution, and radioactivity was measured using a liquid scintillation counter (Aloka). Specific binding was calculated by subtracting the nonspecific binding from the total binding. Non-specific binding was measured in the presence of unlabelled 10$^{-4}$ M cimetidine.

4) Drugs

[N-methyl-3H]-cimetidine (23.0 Ci/mmol) was obtained from Amersham (England). Histamine, 3-isobutyl-1-methyl xanthine (IBMX), bovine serum albumin (fraction V), acridine orange and N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from Sigma (St. Louis, U.S.A.) and collagenase (type IV) from Worthington (Freehold, U.S.A.). Cimetidine, etintidine, ranitidine, TZU-0460 and famotidine were gifts from Smith, Kline & Fujisawa (Osaka, Japan), Banyu (Tokyo, Japan), Glaxo (Ware, England), Teikoku (Kawasaki, Japan) and Yamanouchi (Tokyo, Japan), respectively.

5) Data analysis

Results are expressed as means±S.D. or means with 95% confidence limits. IC$_{50}$ values and a pseudo Hill coefficient ($n_\text{H}$) for the inhibition curves in $^3$H-cimetidine binding were calculated from least-squares non linear regression analysis, using a one site model (15). The Scatchard plot calculated from cimetidine inhibition data was analyzed by least-squares non-linear regression analysis, according to Feldman (16). ED$_{50}$ values for histamine dose-response curves with cellular cAMP were calculated from logit-log analysis (17) followed by linear regression analysis. The proportion of specific $^3$H-cimetidine binding to the high affinity site is calculated on the assumption that both high and low affinity sites obey the simple mass action law (18).

Results

1) $^3$H-Cimetidine binding studies: To study the effects of cations on $^3$H-cimetidine binding, five chloride salts were tested. Of the five chloride salts (CaCl$_2$, CuCl$_2$, MgCl$_2$, KCl, NaCl), CuCl$_2$ remarkably increased specific $^3$H-cimetidine binding (Fig. 2). In the presence of 50 nM Cu$^{2+}$, the specific $^3$H-cimetidine binding to the membrane preparations was stable and maximal at 4°C and reached equilibrium within 40 min. On the other hand, the specific $^3$H-cimetidine binding at 37 and 22°C did not reach the same degree observed by incubation at 4°C for 40 min, and it decreased with the passage of time (Fig. 3). Therefore, subsequent assays were performed at 4°C for 60 min in the presence of 50 nM Cu$^{2+}$. The binding of $^3$H-cimetidine to the membrane preparations was reversible by the addition of an excess of unlabelled cimetidine, and boiling the membranes for 10 min prior to the binding study reduced specific $^3$H-cimetidine binding to about one-tenth.

Inhibition by unlabelled cimetidine of $^3$H-cimetidine binding gave a rather flat curve with a pseudo Hill coefficient of less than unity ($n_\text{H} 0.76±0.10; n=6$), and it resulted in a hyperbolic Scatchard plot which could be separated into two linear components, as-

![Fig. 2. Effect of Cu$^{2+}$ on $^3$H-cimetidine binding in membranes of isolated guinea pig gastric glands. Membranes were incubated with $^3$H-cimetidine (8 nmol/l) in the presence of varying concentrations of Cu$^{2+}$ at 4°C for 60 min. Cu$^{2+}$ was added to the incubation medium as CuCl$_2$. The height of each bar represents total binding expressed as pmol/mg protein. Non-specific binding was measured in the presence of unlabelled cimetidine (10$^{-4}$ M). Values are the means of three experiments.](image)
Fig. 3. Effects of incubation time and temperature on specific $^3$H-cimetidine binding in membranes of isolated guinea pig gastric glands. Membranes were incubated with $^3$H-cimetidine (9 nM) in the presence of 50 nM CuCl$_2$ at each temperature. Specific binding was calculated by subtracting the non-specific binding measured in the presence of unlabelled cimetidine ($10^{-4}$ M) from total binding. Data are expressed as pmol/mg protein. Values are the means of three experiments.

suming two classes of binding sites: high affinity and low affinity (Fig. 4). The high affinity binding site had a $K_d$ of 0.41±0.1 x10$^{-6}$ M and a $B_{max}$ of 265±67 pmol/mg protein, while the low affinity binding site had a $K_d$ of 12.8±4.6 x10$^{-6}$ M and a $B_{max}$ of 4,030±880 pmol/mg protein. For comparative purposes, a Scatchard plot of the inhibition of $^3$H-cimetidine by unlabelled cimetidine at 37°C for 10 min ($n=3$) was prepared. Two components could be distinguished. At a temperature of 37°C, $K_d$ and $B_{max}$ values for the high affinity binding site were 0.34±0.29 x10$^{-6}$ M and 32.8±26 pmol/mg protein, and those for the low affinity binding site were 33.1±14.0 x10$^{-6}$ M and 4,128±394 pmol/mg protein, respectively. In comparison with the study at 4°C, while the $K_d$ value for the high affinity binding site at 37°C was similar, the $B_{max}$ value for the high affinity binding site at 37°C markedly decreased.

Competition experiments were performed with a low concentration of $^3$H-cimetidine (9 nM) at 4°C, and the potencies of histamine, several newer H$_2$-receptor antagonists (etini-
didine, ranitidine, TZU-0460 and famotidine), two H$_1$-receptor antagonists (mepyramine, diphenhydramine), atropine, imidazole and clonidine, an imidazoline $\alpha_2$-adrenoceptor agonist, to inhibit specific $^3$H-cimetidine

Fig. 4. Inhibition of $^3$H-cimetidine binding to membranes of guinea pig isolated gastric glands by unlabelled cimetidine. Membranes were incubated with $^3$H-cimetidine (9 nM) and 50 nM CuCl$_2$ in the presence of increasing concentrations of unlabelled cimetidine at 4°C for 60 min. Data are expressed as percentage of the specific binding obtained with the tracer alone. Specific binding was calculated by subtracting the non-specific binding measured in the presence of unlabelled cimetidine ($10^{-4}$ M) from the total binding (inset). Scatchard representation of these data obtained after correcting the specific activity of $^3$H-cimetidine for the unlabelled cimetidine added. The hyperbolic Scatchard plot was separated into two linear components, assuming two classes of binding sites (main figure). Values are the means of six experiments.
Fig. 5. Inhibition of specific $^3$H-cimetidine binding to membranes of isolated guinea pig gastric glands by histamine, $H_1$-receptor antagonists, $H_2$-receptor antagonists and other compounds. Membrane preparations were incubated with $^3$H-cimetidine (9 nM) in the presence of 50 $\mu$M CuCl$_2$ at 4°C for 60 min. Data are expressed as a percentage of the specific binding obtained with the tracer alone. Specific binding was calculated by subtracting the non-specific binding measured in the presence of unlabelled cimetidine (10$^{-4}$ M) from total binding. Values are means of 3–6 experiments.

Table 1. Concentrations (with 95% confidence limits) of the drugs required for a half maximal inhibition of specific $^3$H-cimetidine binding (apparent IC50 values)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Apparent IC50 (M)</th>
</tr>
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<tbody>
<tr>
<td>Histamine</td>
<td>6.8 (5.0–8.6)$\times$10$^{-6}$</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>1.8 (1.6–2.0)$\times$10$^{-6}$</td>
</tr>
<tr>
<td>Etintidine</td>
<td>5.0 (2.1–7.9)$\times$10$^{-7}$</td>
</tr>
<tr>
<td>TZU-0460</td>
<td>$&gt;10^{-3}$</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>$&gt;10^{-3}$</td>
</tr>
<tr>
<td>Famotidine</td>
<td>4.4 (4.0–4.8)$\times$10$^{-6}$</td>
</tr>
<tr>
<td>Mepyramine</td>
<td>$&gt;10^{-3}$</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>6.9 (3.7–10.2)$\times$10$^{-4}$</td>
</tr>
<tr>
<td>Clonidine</td>
<td>$&gt;10^{-3}$</td>
</tr>
<tr>
<td>Imidazole</td>
<td>3.3 (3.1–3.5)$\times$10$^{-4}$</td>
</tr>
</tbody>
</table>

Data were obtained from the same experiments as Fig. 5.

binding were determined as apparent IC50 values (Fig. 5, Table 1). Cimetidine and etintidine were potent inhibitors of $^3$H-cimetidine binding, while ranitidine and famotidine were weak inhibitors. TZU-0460 showed little inhibition, even at high concentrations. The relative potencies of these antagonists were etintidine $>$ cimetidine $>$ famotidine $>$ ranitidine $>$ TZU-0460. If the potency of cimetidine to inhibit $^3$H-cimetidine binding is assigned a value of 100, the relative potencies were as follows: cimetidine, 100; etintidine, 360; and famotidine, 4 (calculated from apparent IC50 values).

2) Cellular cAMP studies: To explore the correlation between antagonist affinity found in the binding study and antagonist potency obtained in pharmacological tests, the effects of $H_2$-antagonists on the histamine-stimulation of cellular cAMP were investigated. Histamine caused a 11.7-fold increase in cellular cAMP (basal 4.3±0.9 pmol/mg protein, maximum 49.7±4.4 pmol/mg protein), with a half maximal increase occurring at 6.3$x10^{-5}$ M histamine (n=16). Cimetidine did not alter cAMP in control glands, but caused a parallel rightward shift in the dose response curve for histamine, and with a sufficiently high concentration of histamine, the inhibition caused by cimetid-
dine was abolished, thereby indicating a simple competitive antagonism. The shift of the dose-response curve for histamine was analyzed by the method of Arunlakshana and Schild (19). The slope of the regression line (with the 95% confidence limits) was 0.99 (0.86–1.11) (Fig. 6). Similar results were obtained with etintidine, ranitidine, famotidine and TZU-0460. All antagonists showed competitive inhibition against histamine-stimulation of cellular cAMP, since the slopes of the regression line describing log (DR-1), as a function of log antagonist concentration, were not significantly different from unity. Furthermore, the calculated affinities of the newer antagonists for the receptor were

![Histamine cumulative log-dose response curves from cellular cAMP in isolated guinea pig gastric glands. Gastric glands were stimulated by varying concentrations of histamine (plus 10^-5 M IBMX) without cimetidine (○—○) or with cimetidine: 4.1 X 10^-7 M (▲—▲), 1.2 X 10^-6 M (■—■), 3.7 X 10^-6 M (△—△) and 1.1 X 10^-5 M (□—□). Values are means of five experiments. Same results expressed as log (DR-1) against log cimetidine concentration (inset). The dose ratio (DR) were calculated from the ratio of the ED50 (the concentration required for a half maximal response) values for histamine in the presence versus the absence of various concentration of cimetidine. X-intercept gives a pA2 value.

Table 2. Comparison of the parameters obtained from Schild plot analysis for the effect of histamine H2-receptor antagonists on the histamine-stimulation of cellular cAMP

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Slope</th>
<th>pA2</th>
<th>Kᵦ (nM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cimetidine</td>
<td>0.99 (0.86–1.11)</td>
<td>6.41 (6.30–6.53)</td>
<td>0.39 (0.30–0.50)</td>
<td>5</td>
</tr>
<tr>
<td>Etintidine</td>
<td>1.07 (0.96–1.18)</td>
<td>6.82 (6.76–6.88)</td>
<td>0.15 (0.13–0.17)</td>
<td>4</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>1.05 (0.91–1.18)</td>
<td>6.87 (6.77–6.97)</td>
<td>0.14 (0.11–0.17)</td>
<td>3</td>
</tr>
<tr>
<td>TZU-0460</td>
<td>1.02 (0.78–1.25)</td>
<td>6.94 (6.79–7.09)</td>
<td>0.11 (0.08–0.16)</td>
<td>5</td>
</tr>
<tr>
<td>Famotidine</td>
<td>1.04 (0.96–1.13)</td>
<td>7.60 (7.51–7.69)</td>
<td>0.03 (0.02–0.03)</td>
<td>4</td>
</tr>
</tbody>
</table>

Gastric glands were stimulated by varying concentrations of histamine (plus 10^-5 M IBMX), with or without H2-receptor antagonists. The concentrations of H2-receptor antagonists tested were 1.4 X 10^-7 M, 4.1 X 10^-7 M, 1.2 X 10^-6 M for etintidine, ranitidine and TZU-0460, and 4.6 X 10^-6 M, 1.4 X 10^-7 M, 4.1 X 10^-7 M and 1.2 X 10^-6 M for famotidine. Values were computed as described in the legend of Fig. 6, and 95% confidence limits are given.
higher than that of cimetidine (Table 2). If the potency of cimetidine in inhibiting the histamine-stimulation of cellular cAMP is assigned a value of 100, the relative potencies of these antagonists were as follows: cimetidine, 100; etintidine, 250; ranitidine, 280; TZU-0460, 360; and famotidine, 1300 (calculated from $K_B$ values).

**Discussion**

We have demonstrated specific $^3$H-cimetidine binding sites in membrane preparations of isolated guinea pig gastric glands in the presence of Cu$^{2+}$, at concentrations similar to those in the blood (50 μM) (11). Greenaway et al. (20) reported that cimetidine reacts with Cu$^{2+}$ to produce the Cu$^{2+}$-cimetidine complexes which may increase specific cimetidine binding. Specific $^3$H-cimetidine binding was maximal and stable at 4°C. For this reason, in the present study, we studied the reversibility, saturability and drug specificity of $^3$H-cimetidine binding at a temperature of 4°C, although ligand binding studies should be done at a temperature close to 37°C. Non-specific binding was measured in the presence of unlabelled 10$^{-4}$ M cimetidine. $^3$H-Cimetidine binding was no longer decreased by unlabelled cimetidine over the concentration of 10$^{-4}$ M when the concentration of $^3$H-cimetidine was 9 nM. Scatchard plot analysis revealed two classes of binding sites. To obtain apparent IC50 values for drugs in $^3$H-cimetidine binding to the high affinity sites, competition experiments were performed with a low concentration of $^3$H-cimetidine (9 nM). Under these conditions, $^3$H-cimetidine binding to the high affinity site was estimated from saturation data to represent 67% of the total specific binding, and imidazole-derived H$_2$-antagonists (cimetidine and etintidine) were most potent in inhibiting $^3$H-cimetidine binding, and histamine and famotidine were more potent than other drugs. A very potent and specific agonist for histamine H$_2$-receptor has been synthesized (8), but the only H$_2$-receptor agonist used in the present study was histamine.

As a physiological or pharmacological response is essential for defining a receptor, experiments should be done to correlate the drug affinity found in the binding study with the drug potency obtained in pharmacological tests, performed either in vivo or in vitro. The responses mediated by the histamine H$_2$-receptor are atrial contraction, relaxation of the rat uterus and stimulation of gastric acid secretion (1), and discrepancies between the affinities for the H$_2$-receptor antagonists in various H$_2$-receptor preparations and animals has been reported (2, 21). Because of the difficulty in directly measuring acid secretion from isolated gastric glands or parietal cells, changes in cellular cAMP, as an index for the acid secretory response, were used in the present study. Gastric glands are composed of a few types of cells. Berglindh and Oblink (22) also reported that four types of cells could be identified among isolated rabbit gastric glands: parietal cells, zymogen cells, mucous neck cells and some endocrine cells. Although dispersed gastric mucosal cells or isolated gastric glands contain, more or less, non parietal cells, the histamine stimulated adenylate cyclase system is presumed to be located in the parietal cell component (23, 24). We used five H$_2$-receptor antagonists, all with different structures, i.e., cimetidine and etintidine (an imidazole derivative), ranitidine (a furan derivative) famotidine (a thiazole derivative) and TZU-0460 (a benzene derivative). TZU-0460, in particular, differs considerably in chemical structure from other H$_2$-receptor antagonists. Although it has been suggested that the critical structural feature of the H$_2$-receptor antagonist is the imidazole ring (7) or ethylthio-methyl ring-side chain of various guanidine derivatives (8), TZU-0460 has neither of these components (Fig. 1).

Rising et al. (11) suggested that $^3$H-cimetidine might not label the H$_2$-receptor in the guinea pig cerebral cortex and gastric mucosa membrane preparations. In their experiments, the $K_B$ values for $^3$H-cimetidine did not correspond to the $K_B$ value for cimetidine, calculated from pharmacological tests. Gillian et al. (13) reported that $^3$H-tiotidine met the criteria for labelling of the H$_2$-receptor in the guinea pig cerebral cortex, but they could not demonstrate specific $^3$H-tiotidine binding in other tissues regarded as having H$_2$-receptors (guinea pig gastric
mucosa and right atrium and rat uterus and cerebral cortex). Maayani et al. (25) also failed to demonstrate specific $^3$H-tiotidine binding in the rat hippocampus and postulated that $^3$H-tiotidine is useless for labelling the H$_2$-receptor in a binding study. On the other hand, Kendall et al. (10) reported that in the presence of Cu$^{+}$, $^3$H-cimetidine might be labelling a biologically relevant H$_2$ binding site in rat brain membrane fractions. In our experiments, the $K_b$ value for cimetidine was close to the $K_d$ value for the high affinity $^3$H-cimetidine binding site, and the relative potency of etintidine against cimetidine in inhibiting $^3$H-cimetidine binding is similar to the relative potency of etintidine against cimetidine in inhibiting $^3$H-cimetidine binding. Although Smith et al. (12) concluded that the $^3$H-cimetidine binding site in rat brain tissue is not the histamine H$_2$-receptor, because of the lack of inhibition observed by non-imidazole H$_2$-receptor antagonists, we suggest a different hypothesis. Binding subunits of the H$_2$-receptor may explain the weak inhibition of $^3$H-cimetidine binding by non-imidazole H$_2$-receptor antagonists.

Multiple binding sites for agonists and antagonists on the acetylcholine receptor have been postulated (26). With respect to the histamine H$_2$-receptor, Hersey (27) suggested that the H$_2$-receptor molecule has at least three critical binding sites for agonists and antagonists: whereas histamine binds to all sites, other agonists do not. Weinstein et al. (28) assumed that the protonated amine side chain of histamine binds to the anionic site I of the H$_2$-receptor and the imidazole ring to sites II and III by hydrogen bonding. In such a receptor model with multiple binding sites for an agonist, it may be that the binding site for one agonist is not identical with that for another antagonist of a different chemical structure. Bristow et al. (9) reported that even at high concentrations, cimetidine could not inhibit $^3$H-ranitidine binding and ranitidine could not inhibit $^3$H-cimetidine binding in the guinea pig heart membrane preparations. The present study suggests that histamine may bind to the H$_2$-receptor of gastric glands at several sites and that imidazole-derived H$_2$-receptor antagonists and non-imidazole derived H$_2$-receptor antagonists compete with histamine at different sites on the H$_2$-receptor.

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