Characterization of I₂ Imidazoline and σ Binding Sites in the Rat and Human Stomach

GERHARD J. MOLDERINGS, KURT DONECKER, MARIA BURIAN, W. ALEXANDER SIMON, DETLEV W. SCHRÖDER and MANFRED GÖTHERT

Institute of Pharmacology and Toxicology (G.J.M., K.D., M.B., M.G.), University of Bonn, Bonn, Germany, Byk Gulden Lomberg GmbH (W.A.S.), Konstanz, Germany and Evangelisches Krankenhaus (D.W.S.), Bonn, Germany

Accepted for publication December 1, 1997 This paper is available online at http://www.jpet.org

ABSTRACT

Radioligand binding experiments were carried out to identify and characterize nonadrenoceptor [³H]idazoxan binding sites and [³H](1,2-di-(2-toly)guanidine) binding sites in the rat and human stomach. Furthermore, we examined two selected aspects of their potential functional significance. Binding of [³H]idazoxan (Kᵦ = 11.1 nM and 12.4 nM, respectively) and [³H]DTG (Kᵦ = 932 nM and 242 nM, respectively) to cell membranes from rat and human stomach was rapid, reversible, specific and saturable. In rat stomach, binding of the radioligands was inhibited by imidazolines and by nonimidazoline σ-site ligands, respectively, at different rank orders of affinity, which suggests the existence of I₂-imidazoline binding sites as well as σ₂-sites. In two functional models, the direct effects of I₂-site ligands and σ₂-site ligands on gastric smooth muscle and glands were investigated. (1) Cirazoline, clonidine and 4-chloro-2-(2-imidazolin-2-ylamino)-isoindoline (BDF 6143) failed to contract the longitudinal muscle of the rat stomach fundus; BDF 6143 also failed to induce relaxation of this preparation when it was precontracted with 30 mM KCl. (2) Clonidine, idazoxan, BDF 6143, 1,2-di-(2-toly)guanidine, agonist and (R)-3-(3-hydroxyphenyl)-N-propylpiperidine up to 100 μM did not induce acid secretion from rabbit isolated gastric glands. Our data provide evidence that the rat stomach is endowed with σ₂-sites and I₁-sites in addition to the previously identified non-I₁/non-I₂ [³H]clonidine binding sites. Our experiments also offer basic evidence of the existence of I₂ and σ binding sites in the human stomach. Neither the I₂-clonidine binding sites nor the σ-sites in rat stomach are directly related to a postsynaptic effect on gastric smooth muscle or to acid release from isolated gastric glands.

Nonadrenoceptor IBS are recognized with high to moderate affinity by imidazolines and related compounds, but not by catecholamines. At least two classes of IBS exist, I₁-IBS and I₂-IBS, which can be labeled by [³H]clonidine and [³H]idazoxan, respectively (for review, see Regunathan and Reis, 1996; Molderings, 1997). Binding experiments with [³H]clonidine and [³H]idazoxan in membranes from guinea pig (Hou et al., 1987), rabbit (Tesson et al., 1992) and rat gastric tissue (Molderings et al., 1995) provided basic evidence that IBS are also present in the stomach. Interestingly, it has been shown that in the rat stomach, σ-receptor ligands exhibited a remarkably high affinity for nonadrenoceptor [³H]clonidine binding sites (Molderings et al., 1995). Moreover, σ-like sites were recently identified in the porcine gastric mucosa (Harada et al., 1994), but not in rat and human gastric tissue.

On the basis of these findings, the first aim of the present study was to identify and characterize [³H]idazoxan and [³H]DTG binding sites in the rat and human stomach and to investigate whether a relationship exists between nonadrenoceptor [³H]clonidine and [³H]idazoxan binding sites on the one hand and σ binding sites on the other. Therefore, we determined and compared the affinity of key ligands for IBS and σ sites in rat stomach membranes labeled with [³H]clonidine, [³H]idazoxan or [³H]DTG (a radioligand for σ sites; Weber et al., 1986).

In previous in vivo studies, imidazolines such as clonidine exerted a dual action on gastric acid secretion; at low concentrations, these compounds reduced acid secretion (Del Tacca et al., 1982; Bhandare et al., 1991; Blandizzi et al., 1995; Carlisle et al., 1995; Glavin and Smyth, 1995). This inhibitory effect on acid secretion was prevented by alpha-2 adrenoceptor antagonists (Del Tacca et al., 1982; Bhandare et al., 1991; Blandizzi et al., 1995), which suggests that it is mediated mainly by activating presynaptic alpha-2 adrenoceptors on cholinergic nerves innervating the stomach. Additionally, evidence has been presented that peripheral I₁-imidazoline receptors might also contribute to the antisecretory and an...
tiulcer effects of imidazoline derivatives (Carlisle et al., 1995; Glavin and Smyth, 1995). At higher concentrations, several imidazolines and agmatine stimulated acid secretion in vitro and in vivo (Medgett and McCulloch, 1979; Del Tacco et al., 1982; Houi et al., 1987; Bhandare et al., 1991; Glavin et al., 1995). The stimulatory effect was not due to activation of alpha-2 adrenoceptors, because it was not mimicked by the alpha-2 adrenoceptor agonist a-methylnoradrenaline and it was not counteracted by yohimbine (Houi et al., 1987). Some have speculated that the stimulatory effect of the imidazolines may be due to activation of imidazoline receptors in stomach tissue (Houi et al., 1987; Bhandare et al., 1991; Glavin et al., 1995). Therefore, the second aim of this study was to investigate whether ligands with high affinity for IBs and/or sites could induce acid secretion from isolated rabbit gastric glands, which is the standard preparation for investigating acid secretion in vitro.

Finally, it was demonstrated that CDS, a putative endogenous ligand at IBs (Regunathan and Reis, 1996) induced a contraction of gastric smooth muscle via an unknown mechanism of action (Felsen et al., 1987). It has been proposed that imidazoline receptors on gastric smooth muscle may be involved. Hence the third aim of the present study was to examine whether other ligands at imidazoline binding sites mimic the effect of CDS and, if so, whether binding of drugs at [3H]clonidine, [3H]Iodoxazan and [3H]DTG binding sites directly contract rat stomach smooth muscle cells in a vagal nerve-independent manner. Parts of this study have been presented at scientific meetings.

Materials and Methods

Membrane preparation. Fresh stomachs were obtained from Wistar Kyoto rats immediately after killing. Segments of macroscopically normal human stomach were obtained from male or female patients undergoing gastric surgery. The study was approved in all respects by the local ethics committee. All steps of the preparation procedure were performed on ice. The rat glandular stomach and the segments of the mucosal layer from human stomach were prepared and cut into small fragments that were placed in 40 ml of buffer solution containing sucrose 270 mM, ascorbic acid 0.6 mM and Tris-sulfate 10 mM (pH 7.4), mixed by means of an Ultraturrax (five times for 20 s each) and homogenized using a glass-Teflon homogenizer (three times for 30 s each). The homogenates were centrifuged (5 min, 1200 × g, 4°C). The supernatant was filtered through four layers of gauze, diluted to 420 ml with HEPES buffer (HEPES-Na 5 mM, EGTA 0.1 mM, PMSF 0.3 mM, pH 7.4; buffer I) and re-centrifuged (20 min, 40,000 × g, 4°C). The pellet was washed twice and then resuspended in buffer I, homogenized, diluted to give a protein concentration of about 2 mg/ml and stored at −80°C until use. Before use, the membranes were centrifuged (20 min, 40,000 × g, 4°C), resuspended in the incubation buffer (HEPES-Na 5 mM, EGTA 0.5 mM, MgCl2 0.5 mM, ascorbic acid 0.1 mM, pH 7.4; buffer II), homogenized by ultrasonics and diluted to a final protein concentration of about 0.6 mg/ml.

Binding assay. A 400-μl aliquot of membranes was incubated for 55 min with [3H]Iodoxazan or [3H]DTG (25 μM) at 4°C in a final volume of 0.5 ml. The reaction was stopped by rapid vacuum filtration with a Brandel cell harvester through Whatman GF/C glass-fiber filters presoaked with polyethyleneimine 0.5 M and clonidine 0.1 mM (to reduce filter binding), followed by rapid washing (within about 5 s) of the incubation tubes and filters with 10 ml ice-cold buffer II. Filters were placed in 6 ml of scintillation fluid and shaken overnight, and the radioactivity was determined by liquid scintillation counting at 44% efficiency. Nonspecific binding was defined as radioligand binding in the presence of BDF 6143 100 μM ([3H]Iodoxazan) or in the presence of (+)-3-PPP 100 μM ([3H]DTG binding), and it accounted for 5% and 21% of the total radioactivity retained in the filters when [3H]Iodoxazan and [3H]DTG 10 nM were used in the competition experiments, respectively. Adrenaline 10 μM, which has no affinity for imidazoline binding sites (Molderings et al., 1993, 1994) was added to the assay to prevent the radioligands from binding to alpha-2 adrenoceptors.

In rat stomach, saturation studies with [3H]Iodoxazan were performed with radioligand concentrations ranging from 0.1 to 46 nM to determine receptor number and affinity. Because it has been shown that the affinity of [3H]DTG for peripheral σ sites is in the high nanomolar range i.e., a concentration of the radioligand at which it cannot be used because the cost is too high, equilibrium-saturation binding of [3H]DTG was performed by incubating the membranes with 10 nM [3H]DTG and increasing the concentrations of unlabeled DTG for 55 min at 4°C in both rat and human stomach. A similar experimental protocol has been used to determine the affinity and the density of [3H]Iodoxazan binding sites in human stomach. This competitive protocol not only has the advantage over saturation protocols that less radioligand is consumed, but the contribution of nonspecific binding is also minimized because the radioligand is used at a low concentration (DeBlasi et al., 1989). Competition studies were done using 10 nM [3H]Iodoxazan or [3H]DTG, respectively, and 13 different concentrations, ranging from 0.1 nM to 100 μM, of the unlabeled ligand under investigation. All experiments were carried out in triplicate.

Determination of gastric acid release. Gastric glands were prepared from anesthetized New Zealand rabbits by high-pressure perfusion of the stomach followed by collagenase digestion of pieces of fundic mucosa. Gastric glands were suspended in Krebs-Henseleit solution (NaCl 132.5 mM, KCl 5.4 mM, Na2HPO4 5 mM, NaH2PO4 5 mM, MgSO4 1 mM, CaCl2 1.2 mM) containing 2 mg/ml glucose and 0.125 μM [dimethylamine-14C]aminopyrine; pH 7.4. Glands were incubated at 37°C in the presence of the drugs at the concentrations indicated. After 30 min, the glands were sedimented by rapid centrifugation, and the aminopyrine accumulation ratio was determined from the relation of radioactivity between the glands and the supernatant (for details, see Simon et al., 1990).

Contraction of gastric smooth muscle. Gastric longitudinal muscle strips (3 × 10 mm) were obtained from Wistar-Kyoto rats. The stomach, opened along the greater curvature, was stripped free of all mucosal tissue, and longitudinal strips were cut at right angles to the visible circular muscle bundles. In an organ bath, tissue strips were pre-equilibrated for about 1 h at 37°C in oxygenated physiological salt solution of the following composition (mM): NaCl 118, KCl 4.7, CaCl2 2.5, MgCl2 1.2, NaHCO3 25, KH2PO4 1.2, glucose 10; pH 7.4. This solution contained 1 μM atropine, 1 μM prazosin and 3 μM rauwolscine throughout the experiments to mask alpha-adrenoceptors and to eliminate potential vagal nerve influences. Cumulative concentration-response curves were determined for the drugs under study. Time-matched control experiments were carried out in parallel. Contractions were monitored isometrically using Statham force transducers under an load of 1 g. After the last concentration of the test compound, the preparation was washed twice, and then KCl was added to the organ bath (final concentration 85 mM) to induce maximum contraction of the smooth muscles. The responses of the test compounds are expressed as the ratio of the contraction evoked by the test drugs to that caused by 85 mM KCl.

Protein assay. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as the standard.

Data analysis. Data from the saturation and competition experiments were analyzed using the least-squares fitting program GraphPadInPlot (GraphPad Software Inc., San Diego, CA). The significance of the improvement of fit obtained by the two-site equation over the fit obtained by the one-site equation was analyzed by the F statistics (partial F test; De Lean et al., 1982). Receptor
Results

[3H]Idazoxan binding. The specific binding of [3H]idazoxan to rat and human stomach membranes was saturable. Nonlinear regression analysis revealed a reaction of [3H]idazoxan with one binding site (Kd = 11.1 ± 2.2 nM and 12.4 ± 4.7 nM, respectively; Bmax = 139 ± 4 fmol/mg protein and 68 ± 14 fmol/mg protein, respectively; figs. 1 and 2).

In competition experiments, most of the compounds listed in table 1 inhibited specific binding of 10 nM [3H]idazoxan to rat stomach membranes in a concentration-dependent manner; at this radioligand concentration, specific binding amounted to 1188 ± 106 dpm (corresponding to about 95% of total binding; n = 48). Competition of BDF 6143 with [3H]idazoxan 10 nM revealed an inhibition curve with a slope factor nH of less than 1. Accordingly, the competition curve for this compound was significantly better fitted to a two-site than to a one-site model (table 1; fig. 3). With the other competitors, monophasic displacement curves were obtained (nH was not significantly different from 1.0; fig. 3). The Kd values at the high-affinity site or the single site for all drugs investigated ranged from 11 to 265,800 nM (table 1) with the following rank order of affinities: idazoxan > cirazoline > BDF 6143 > naphazoline > dioxizoline > (+)-3-PPP > (-)-ifenprodil = clonidine > (+)-pentazocine > agmatine. Histamine, rauwolscine and ranitidine at concentrations up to 100 μM inhibited binding by less than 50% (table 1).

[3H]DTG binding. The specific binding of [3H]DTG to rat and human stomach membranes was saturable. Homologous displacement experiments with unlabeled DTG and [3H]DTG revealed a reaction of [3H]DTG with one binding site with Kd values of 932 ± 319 nM and 242 ± 90 nM, respectively, and Bmax values of 7087 ± 4024 fmol/mg protein and 3592 ± 553 fmol/mg protein, respectively (figs. 2 and 4). In competition experiments, the compounds listed in table 2 inhibited specific binding of [3H]DTG 10 nM to rat stomach membranes in a concentration-dependent manner; at this radioligand concentration, specific binding amounted to 1206 ± 75 dpm (corresponding to 79% of total binding; n = 56). The drugs investigated as competitors of [3H]DTG revealed monophasic displacement curves (nH was not significantly different from 1; fig. 5). The Kd values of the compounds ranged from 0.5 to 668 μM (Table 2). The rank order of affinities was as follows (table 2): haloperidol ≥ (-)-ifenprodil ≥ DTG ≥ BDF 6143 ≥ clonidine ≥ (+)-3-PPP ≥ cirazoline ≥ naphazoline ≥ idazoxan ≥ moxonidine ≥ agmatine.

Contraction of rat gastric fundus strips. In timematched control experiments with the vehicle applied, the tension of the preparation slightly decreased by about 2% to 14% (not shown). The thromboxane analog U46619 induced a concentration-dependent contraction of the rat gastric longitudinal muscle strips (fig. 6). In contrast, the imidazolines cirazoline, clonidine and BDF 6143 up to 100 μM (the highest concentration investigated, fig. 6) failed to elicit a contractile response. When the smooth muscle preparation was precon-
Affinities ($K_v$ values) of imidazolines and other compounds for $[^3H]$idazoxan binding sites in rat stomach membranes

Results from computer analysis of competition curves obtained by adding various concentrations of a competing ligand and a fixed concentration (10 nM) of $[^3H]$idazoxan. The percentages of high (%high) and low (%low) affinity sites are given for BDF 6143, which was best resolved (partial $F$ test, last column) into a two-site fit. In parenthesis is the number of experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{\text{high}}$ (nM)</th>
<th>%high</th>
<th>$K_{\text{low}}$ (nM)</th>
<th>%low</th>
<th>$n_H$</th>
<th>Partial $F$ test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idazoxan (3)</td>
<td>11</td>
<td>100</td>
<td>7700</td>
<td>30</td>
<td>-0.98</td>
<td></td>
</tr>
<tr>
<td>Cirazoline (4)</td>
<td>21</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>-1.02</td>
<td></td>
</tr>
<tr>
<td>BDF 6143 (4)</td>
<td>29</td>
<td>70</td>
<td>30</td>
<td>1.36</td>
<td></td>
<td>0.012</td>
</tr>
<tr>
<td>Naphazoline (6)</td>
<td>41</td>
<td>100</td>
<td>100</td>
<td>1.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicoziparine (4)</td>
<td>10500</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-0.87</td>
<td></td>
</tr>
<tr>
<td>(+)-3-PPP (3)</td>
<td>12200</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-0.92</td>
<td></td>
</tr>
<tr>
<td>(±)-Ifenprodil (4)</td>
<td>13400</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-0.92</td>
<td></td>
</tr>
<tr>
<td>Clonidine (3)</td>
<td>13900</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-0.92</td>
<td></td>
</tr>
<tr>
<td>(±)-Pentazocine (7)</td>
<td>165700</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-0.97</td>
<td></td>
</tr>
<tr>
<td>Agmatine (7)</td>
<td>265800</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-0.87</td>
<td></td>
</tr>
<tr>
<td>Histamine (4)</td>
<td>&gt;100000</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rauwolscine (7)</td>
<td>&gt;100000</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ranitidine (5)</td>
<td>&gt;100000</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^*$ A logistic function was fitted to average competition data to obtain slope factors (Hill coefficients, $n_H$ and IC$_{50}$ values). $K_v$ values were then calculated from the IC$_{50}$ values, the $K_v$ value of $[^3H]$idazoxan and the $[^3H]$idazoxan concentration used (Cheng and Prusoff, 1973).

---

**TABLE 2**

Affinities ($K_v$ values) of imidazolines and other compounds for $[^3H]$DTG binding sites in rat stomach membranes

Results from computer analysis of competition curves obtained by adding various concentrations of a competing ligand and a fixed concentration (10 nM) of $[^3H]$DTG. In parenthesis is the number of experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_v$ (µM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloperidol (5)</td>
<td>0.5</td>
<td>-0.87</td>
</tr>
<tr>
<td>(±)-Ifenprodil (4)</td>
<td>0.83</td>
<td>-1.08</td>
</tr>
<tr>
<td>DTG (4)</td>
<td>0.93</td>
<td>-0.86</td>
</tr>
<tr>
<td>BDF 6143 (7)</td>
<td>2.30</td>
<td>-0.87</td>
</tr>
<tr>
<td>Clonidine (5)</td>
<td>2.52</td>
<td>-0.90</td>
</tr>
<tr>
<td>(+)-3-PPP</td>
<td>11.6</td>
<td>-0.90</td>
</tr>
<tr>
<td>Cirazoline (5)</td>
<td>23.2</td>
<td>-0.98</td>
</tr>
<tr>
<td>Naphazoline (4)</td>
<td>26.6</td>
<td>-1.37</td>
</tr>
<tr>
<td>Idazoxan (5)</td>
<td>30.2</td>
<td>-1.05</td>
</tr>
<tr>
<td>Moxonidine (5)</td>
<td>346</td>
<td>-1.62</td>
</tr>
<tr>
<td>Agmatine (7)</td>
<td>668</td>
<td>-1.05</td>
</tr>
</tbody>
</table>

$^*$ A logistic function was fitted to average competition data to obtain slope factors (Hill coefficients, $n_H$ and IC$_{50}$ values). $K_v$ values were then calculated from the IC$_{50}$ values, the $K_v$ value of $[^3H]$DTG and the $[^3H]$DTG concentration used (Cheng and Prusoff, 1973).

---

**Fig. 3.** Competition of imidazoline derivatives (upper panel) and of $\sigma$-site ligands (lower panel) with $[^3H]$idazoxan for its specific binding sites in rat stomach membranes. $\Box$ idazoxan, $\bigcirc$ BDF 6143, $\triangle$ clonidine, $\bigtriangleup$ (+)-3-PPP, $\bullet$ (±)-pentazocine, $\blacktriangle$ (±)-ifenprodil. Each point is the mean of 3 to 7 experiments performed in triplicate. S.E. amounted to up to 11% of the respective mean.

**Fig. 4.** Competition of unlabeled DTG with $[^3H]$DTG for its specific binding sites in rat stomach membranes. Each point is the mean of five experiments performed in triplicate. In some cases, S.E. was smaller than the symbols. At 10 nM $[^3H]$DTG, nonspecific binding accounted for about 16% of the total radioactivity retained in the filters.
induced [14C]aminopyrine accumulation (n = 3; results not shown).

Discussion

[3H]idazoxan binding. Binding of [3H]idazoxan to rat and human stomach membranes was reversible (not shown), specific, saturable and of high affinity; i.e., the criteria for the identification of a specific binding site are fulfilled. The $K_d$ values of [3H]idazoxan (11 nM and 12 nM, respectively) were identical to the $K_v$ value obtained in the competition experiments with unlabeled idazoxan in rat stomach. The values are in the range reported in the literature for I2-imidazoline binding sites (Wikberg et al., 1992; Regunathan et al., 1993; Miralles et al., 1993; Molderings et al., 1994).

In the competition experiments, a shallow displacement curve ($n_H < 1.0$) was obtained with BDF 6143, but not with the other compounds. The curve of BDF 6143 was fitted best to two binding sites by computer modeling. Shallow inhibition curves with Hill coefficients significantly different from unity have also been found in previous studies on bovine chromaffin cells (Molderings et al., 1993; Regunathan et al., 1993) and on guinea pig kidney (Wikberg et al., 1992). As an explanation, it has been proposed that the IBS may exist in two interconvertible forms (Wikberg et al., 1992; Li et al., 1994). Alternatively, the data might be explained by assuming monomer-dimer equilibria (Strange, 1994), in which BDF 6143 would bind with different preference to the monomer or dimer of the binding site and hence would represent a case of negative cooperativity. Finally, it is conceivable that [3H]idazoxan also labeled an I1-like site and that this accounts for the low Hill slope of BDF 6143. In this case, however, cirazoline and naphazoline, which also possess high affinity for the I1-like sites, should also have yielded a biphasic displacement curve. That did not occur, so this explanation appears unlikely.

The present data exclude the possibility that [3H]idazoxan labeled a histamine receptor, in particular an H2 receptor or an alpha-2 adrenoceptor, because histamine and ranitidine failed to compete with high affinity for the [3H]idazoxan site, and the alpha-2 adrenoceptor antagonist rauwolscine exhibited only very low affinity for [3H]idazoxan sites. In agreement with this, the rank order of potency of the competing drugs (table 1) differs from the rank order expected for binding to one of the alpha-2 adrenoceptor subtypes (Bylund et al., 1994). Moreover, (-)-adrenaline in the high concentration of 10 μM was included in the incubation assay to mask
the alpha-2 adrenoceptor population in the membrane preparation.

The pharmacological properties of the idazoxan binding sites in the rat stomach were compared with those of I1-IBS and I2-IBS in bovine adrenomedullary membranes (Molderings et al., 1993, 1994) and of the nonadrenoceptor \([^3H]\)clonidine binding sites in the same tissue (Molderings et al., 1995). The rank order of affinity of the competing drugs for the I1-IBS, i.e., naphazoline > clonidine = cirazoline > BDF 6143 > idazoxan > phentolamine (Molderings et al., 1993), and in particular the affinity of clonidine, are clearly different from the findings in the present experiments (table 1). Therefore, it is unlikely that the \([^3H]\)idazoxan binding site represents an I2-IBS. The \([^3H]\)idazoxan binding sites are also not identical to the nonadrenoceptor \([^3H]\)clonidine binding sites previously described in the rat stomach (Molderings et al., 1995) for two reasons: (1) The affinities of the imidazolines for the \([^3H]\)idazoxan binding sites were not correlated with their affinities for the \([^3H]\)clonidine binding sites in the rat stomach, possess only weak affinity for the \([^3H]\)idazoxan binding sites. In contrast, the rank order of affinity of competing imidazolines and guanidines at I2-IBS in I2-IBS in bovine adrenomedullary membranes was cirazoline > idazoxan > guanabenz > tolazoline > rilmenidine > clonidine > phenolamine (Molderings et al., 1994) and hence conforms to the rank order found in our experiments. Accordingly, the \([^3H]\)idazoxan binding sites identified here may be characterized as I2-IBS.

**[^3H]DTG binding.** Specific \([^3H]\)DTG binding to the rat and human stomach membranes was saturable, reversible (not shown) and of moderate affinity and high capacity. The \(K_r\) values for \([^3H]\)DTG (932 nM and 242 nM, respectively) were consistent with the values previously observed in rat brain and liver, in guinea pig brain, in porcine stomach and on N1E-115 cells (McCann and Su, 1990; DeHaven-Hudkins and Fleissner, 1992; Harada et al., 1994; Molderings et al., 1996).

The affinities of the compounds for the \([^3H]\)DTG binding sites determined in the competition experiments were in the micromolar to millimolar range, which is compatible with previously published radioligand binding studies of peripheral \(\sigma\) binding sites in porcine stomach (Harada et al., 1994) and on N1E-115 cells (Molderings et al., 1996). Comparison of the \(pK_r\) values of the drugs with their affinities (\(pK_r\) values) for the \(\sigma2\) sites on N1E-115 cells (Molderings et al., 1996) revealed a significant correlation (fig. 8B). These data suggest that the \([^3H]\)DTG binding sites in the rat stomach represent \(\sigma2\) binding sites, which is consistent with the finding of Harada et al. (1994) in pig stomach.

Because the affinities of the imidazolines BDF 6143 and clonidine for these \(\sigma\) sites were in the same range as those of typical \(\sigma\)-site ligands such as haloperidol and DTG (table 2), and because it has recently been shown that nonimidazoline \(\sigma\)-site ligands possess a remarkable affinity for the nonadrenoceptor \([^3H]\)clonidine binding sites in the rat stomach, a relationship between the imidazoline binding sites and the \(\sigma\) sites in the rat stomach was conceivable. However, there was no correlation between the affinity of the drugs for the \([^3H]\)DTG binding sites and their affinity for the nonadrenoceptor \([^3H]\)idazoxan and \([^3H]\)clonidine binding sites in rat stomach (r = 0.15, P > 0.74 and r = 0.10, P > 0.10; seven and nine compounds included in the regression analysis, respectively). Hence the three binding sites appear to represent different entities.

**Functional experiments.** In the stomach, the functional effects of imidazolines and guanidines that have previously been observed were tentatively ascribed to an activation of imidazoline receptors. The present study focused on two clearly nonadrenoceptor-mediated effects: the contraction of gastric smooth muscle and the imidazoline-induced stimulation of gastric acid release. In particular, the question arose whether these effects were related to one of the heretofore-described binding sites at the level of the effector cells.

It was previously reported that CDS, which is assumed to be an endogenous ligand at imidazoline binding sites/receptors (Meeley et al., 1986; Atlas, 1991; Piletz et al., 1995), elicited a concentration-dependent contraction of rat gastric fundus strips (Felsen et al., 1987). Because the CDS-induced contraction was not counteracted by muscarine, bradykinin,
serotonin, angiotensin II, vasopressin or alpha-2 receptor antagonists, it was suggested that the CDS-evoked contraction was mediated by imidazoline receptors (Felsen et al., 1987). However, in the present study BDF 6143, clonidine and cirazoline did not mimic the effect of CDS reported previously, although the compounds possess high affinity for I₁- and/or I₂-imidazoline binding sites/receptors. In contrast, the thromboxan analogue U46619 induced a contraction of the rat gastric strips, which indicates that the smooth muscle cells were viable and able to contract. Hence the binding of the ligands to nonadrenoceptor [³H]idazoxan and [³H]clonidine binding sites in rat stomach did not cause a contraction of gastric smooth muscle. Because in the stomach BDF 6143 and clonidine additionally exhibited an affinity for the [³H]DTG binding sites similar to those of the typical α-site ligands (see table 2), it is also unlikely that these α₂ sites are involved in contraction of gastric smooth muscles. It has also been speculated that the stimulation of gastric acid release induced by high concentrations of clonidine and related compounds in rat and guinea pig (for references, see the Introduction) might be due to activation of imidazoline recognition sites (Houi et al., 1987; Bhandare et al., 1991). Nonadrenoceptor [³H]idazoxan (Tesson et al., 1992) and [³H]clonidine (G.J. Molderings, unpublished results) binding sites have also been found in the rabbit stomach. Therefore, [¹⁴C]aminopyrine accumulation in isolated rabbit gastric glands was used to investigate whether imidazolines increase acid secretion by direct activation of the gastric glands, because this is the standard in vitro model for the study of acid secretory mechanisms of the mammalian parietal cells (Berglindh, 1977; for review see Chew, 1994). As expected, stimulation of H₂ histamine receptors by histamine induced an increase of acid release within the glands and thereby led to an accumulation of the weak base [¹⁴C]aminopyrine (fig. 7). In contrast, BDF 6143, idazoxan and the putative endogenous ligand at imidazoline sites, agmatine (Li et al., 1994), failed to induce an increase in [¹⁴C]aminopyrine accumulation and hence obviously did not induce acid release. Clonidine at the extremely high concentration of 0.1 mM inconsistently increased [¹⁴C]aminopyrine accumulation but was without effect at the lower drug concentrations. The latter finding is in contrast to the increase in acid release induced by clonidine and tolazoline from a guinea pig parietal cell preparation (Houi et al., 1987). This release was ascribed to activation of imidazoline receptors, although the effect was potently inhibited by the H₂ histamine receptor antagonists cimetidine, ranitidine and famotidine (Houi et al., 1987), which, in turn, did not compete with [³H]clonidine for the nonadrenoceptor [³H]clonidine binding sites in guinea pig stomach mucosa (Houi et al., 1987). Because only 65% of the parietal cell preparation prepared by Houi et al. (1987) consisted of parietal cells, a plausible (though speculative) explanation for the discrepancy between our present results and those obtained by Houi et al. might be that the imidazolines stimulate acid release indirectly, perhaps by inducing the release of endogenous histamine. This would be consistent with the observation that the imidazoline-induced release of gastric acid was blocked by H₂ histamine receptor antagonists (Houi et al., 1987). Taken together, our results make it rather unlikely that the stimulatory effect of the imidazolines on gastric acid release observed in vivo and in whole-stomach preparations is due to activation of nonadrenoceptor [³H]idazoxan or [³H]clonidine binding sites located on parietal cells. For the following reasons, we also investigated whether the potent α-site ligands (+)-3-PPP and DTG influenced gastric acid secretion in rabbit glands. (1) α-Site ligands possess a remarkable affinity for the [³H]clonidine binding sites in rat stomach (Molderings et al., 1995). (2) Imidazolines exhibit a considerable affinity for peripheral α₂ sites (Molderings et al., 1996; present study). (3) α₂ Sites are believed to play some role in the control of gastric function (Harada et al., 1994). However, both (+)-3-PPP and DTG failed to increase [¹⁴C]aminopyrine accumulation and, accordingly, did not induce acid secretion by direct stimulation of the parietal cell.

**Conclusion.** Our data provide evidence that, in addition to the previously identified non-I₁/non-I₂ [³H]clonidine binding sites (Molderings et al., 1995), the rat stomach is endowed with [³H]idazoxan binding sites that belong to the I₂ subclass. In addition, α₂ sites are present in the rat stomach. Our experiments also provide basic evidence for the existence of I₂ and σ binding sites in the human stomach. The three different binding sites appear not to act directly on gastric smooth muscle. They also do not mediate direct stimulation of gastric acid release from parietal cells.

**Acknowledgments.**

The technical assistance of Mrs. D. Funcius and Mrs. M. Hartwig is gratefully acknowledged. We wish to thank the pharmaceutical companies for generous gifts of drugs. The study was supported by the Deutsche Forschungsgemeinschaft.

**References.**


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Kᵢ) and the concentration of inhibitor which causes 50 percent inhibition (I₅₀) of an enzyme reaction. Biochem Pharmacol 22:3099–3108.


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Kᵢ) and the concentration of inhibitor which causes 50 percent inhibition (I₅₀) of an enzyme reaction. Biochem Pharmacol 22:3099–3108.


Send reprint requests to: G.J. Molderings, Institute of Pharmacology and Toxicology, University of Bonn, Reuterstr. 2h, D-53113 Bonn, Germany.