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Human Gastric Mucosa Expresses Glandular M3 Subtype of Muscarinic Receptors

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Five subtypes of muscarinic receptors have been distinguished by pharmacological and molecular biological methods. This report characterizes the muscarinic subtype present in human gastric mucosa by radioligand binding studies. The receptor density was 27 ± 6 fmol/mg protein and the tritiated ligand N-*methylscopolamine had an affinity of* (K_D) *0.39 ± 0.08 nM* (n = *11). The M1 receptor selective antagonist pirenzepine and the M2 receptor selective ligand AF-DX 116 had low affinities of 148 ± 32 nM* (n = *13) and 4043 ± 1011 nM* (n = *3)* K_D, *respectively. The glandular M3 antagonists hexahydrosiladifenidol and silahexocyclium had high affinities of* K_D *78 ± 23 nM* (n = *5) and 5.6 ± 1.8 nM* (n = *3). The agonist carbachol interacted with a single low-affinity site and binding was insensitive to modulation by guanine nucleotides. Antagonist and agonist binding studies thus showed an affinity profile typical of M3 receptors of the glandular type.*

KEY WORDS: stomach; human gastric mucosa; muscarinic receptor subtype; acid secretion; glandular M3 receptor.

Five subtypes of muscarinic receptors are presently distinguished based on different primary sequences that were derived from cDNA-clones coding for these receptors (1–9). Muscarinic receptor heterogeneity also has been found by pharmacological methods. M1 receptors mainly are present on neuronal structures and possess a high affinity for pirenzepine (10), telenzepine (11), and *o*-methoxysilahexocyclium (12). M2 receptors occur in cardiac tissue (13, 14) and in intestinal smooth muscle (15–17). They are characterized by a high affinity for ligands like AF-DX 116 (7, 8, 13, 14, 17), and methoctramine (7, 16, 18). M3 receptors (previously termed M2β) display high affinity for silahexocyclium (SiHC), hexahydrosiladifenidol (HHSiD) and related compounds (19, 20, 23) and low affinity with AF-DX 116 and methoctramine (7, 8, 14, 18, 22–24). These receptors were identified in glandular tissue (5, 14, 24) in certain smooth muscle preparations (15–17, 19, 20, 23–25) and in mucosa from porcine stomach (22).

M3 receptors (26) were shown to activate the phosphatidylinositol second messenger system and to mediate muscarinic stimulation of acid secretion in isolated rat gastric parietal cells (26–29). There appear to exist species-specific differences in subtypes of gastric mucosal muscarinic receptors, as rabbits were reported to possess a different subtype with low affinity for HHSiD and AF-DX 116 (30).

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The availability of selective muscarinic antagonists opens new therapeutic possibilities, as specific types of muscarinic receptors may be blocked without the disadvantage of impeding muscarinic function in general (24). A first example is the use of the peripheral M1 antagonist pirenzepine in ulcer therapy. HHSiD was suggested to represent a selective antispasmodic and antisecretory agent without cardiac side effects (24). This study was designed therefore to characterize the subtype of muscarinic receptors occurring in human gastric mucosa by radioligand binding studies.

Experiments were performed using pirenzepine to assess the possible presence of M1 receptors. To further classify the muscarinic receptor subtype present, three antagonists that allow a distinction to be made between M2 and M3 receptors were tested. AF-DX 116 was shown to possess an affinity of about 0.1 μM for cardiac or M2 receptors while its affinity for M3 receptors was 10- to 30-fold lower (7, 8, 13, 14). HHSiD and SiHC have the inverse patterns of affinities with a nanomolar affinity for M3 receptors while their affinity for cardiac receptors is 10- to 30-fold lower (7, 8, 19–21, 26). As a second approach, the affinity of the muscarinic agonist carbachol was determined, which was previously observed to possess 20- to 100-fold lower affinity for M3 as compared to M2 receptors (22, 31, 32). In addition, binding of carbachol to M3 receptors was insensitive to modulation by guanine nucleotides (22), while a characteristic decrease in high-affinity binding, termed GTP-shift, is observed with M2 receptors (31, 32).

MATERIALS AND METHODS

Drugs. Pirenzepine was kindly supplied by Dr. Hammer, Boehringer Ingelheim, Germany. Hexahydrosiladifenidol (HHSiD) and silahexocyclium were synthesized as described (21, 33). AF-DX 116 was a kind gift from Dr. A. Zimmer and Dr. G. Trummlitz, Dr. Karl Thomae GmbH, Germany. Unlabeled *N*-methylscopolamine (NMS) and carbachol were from Sigma (Taufkirchen, Germany). Tris and 5′-guanylylimidodiphosphate (GppNHp) were from Boehringer, Mannheim, Germany. All other reagents were purchased from Merck, Darmstadt, Germany, and were of analytical grade. Tritiated NMS was purchased from Amersham International (specific activity 72 Ci/mmol) or from New England Nuclear Corporation (specific activity 85 Ci/mmol).

Tissue preparation. Human gastric fundic mucosa was obtained on the occasion of surgery performed because of gastric or pancreatic carcinomas or because of recurrent ulcer disease. Resected tissue was placed in ice-cold 0.9% saline. The material used was macroscopically intact fundic gastric mucosa resected by a pathologist as far as possible from a carcinoma. The material was frozen within 30–60 min after resection and stored at −80° C. The tissue used was fundic mucosa as shown by histologic examination after staining with hematoxylin–eosin. Atrophic and markedly gastritic mucosa or tissue showing evidence of infiltration by malignant cells was not used. In the case of antrectomies, material close to the proximal margin, which contained fundic mucosa, was obtained.

On the day of the experiment, the tissue was thawed on ice and subsequent steps were performed at 0–4° C. The mucosa was separated from the submucosa by scraping with a scalpel blade. The scraping was homogenized in 0.32 M sucrose, 5 mM Tris HCl, pH 7.5, in a glass–teflon Potter Elvehjem with 10 up and down strokes of the pestle or with a Polytron Homogenizer at setting 5 for 2 × 10 sec (Bachhofer, Reutlingen, Germany). The homogenate was filtered through two layers of gauze and then centrifuged at 1000 × *g* for 10 min. The pellet was discarded and the supernatant was recentrifuged at 20,000 *g* for 20 min. The resulting pellet was resuspended in 50 mM Tris HCl buffer, pH 7.4, and used for binding assays.

Binding experiments. Equilibrium binding assays were performed by incubation of 0.25 nM tritiated NMS with 8–12 concentrations of unlabeled ligands and the homogenate at 22° C for 60 min in a final vol of 1 ml. Duplicates or triplicates were performed for each point, which differed by less than 10%. Incubations with NMS showed that equilibrium was reached after 35 min and remained constant for at least 2 hr. The incubations were stopped by dilution in ice-cold Tris buffer (50 mM, pH 7.4) to 4 ml in order to promote the rapid dissociation of nonspecifically bound tracer, followed by rapid filtration through glass fiber filter disks (Whatman GF-C), which were washed with 2 × 5 ml of cold Tris buffer. Radioactivity retained by the filters was determined in a β-counter with 4 ml liquid scintillation cocktail at a counting efficiency of 45%. The Lowry method (34) was used for determination of protein with bovine serum albumin as standard. Atropine (1 μM) was used to define nonspecific binding, which was between 10 and 40% of the total tracer bound.

Data analysis. The unmodified counts were analyzed with the Ligand program developed by Munson and Rodbard (35) by nonlinear least-squares regression analysis. Nonspecific binding was treated as a fitted parameter. The best fit model was chosen by using the statistics (*F* test comparing the "goodness of fit" of different models tested, see reference 35) implemented in the program. Results are expressed as means ± SEM and the number of independent experiments (*n*) is given in the text.

RESULTS

Muscarinic receptors in human gastric fundic mucosa were investigated by displacement of tritiated NMS with various unlabeled ligands. NMS was chosen as radioactive ligand because of its

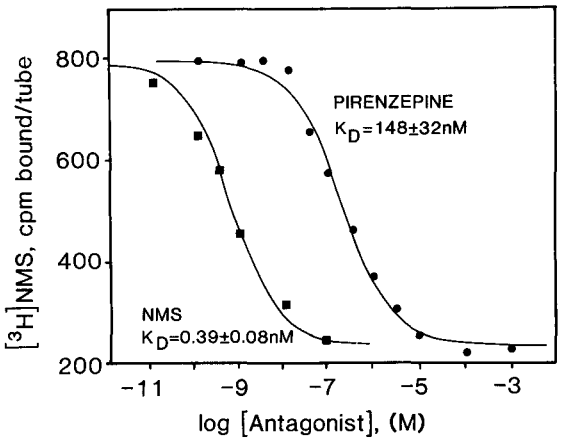

Fig 1. Displacement curves of tritiated *N*-methylscopolamine (NMS) vs unlabeled NMS or pirenzepine in human gastric mucosa. The concentration of [^{3}H]NMS was 0.25 nM corresponding to 18,000 cpm/tube. The data are means from one representative experiment performed in triplicate. The variation was less than 10%. Protein concentration was 1.5 mg/tube.

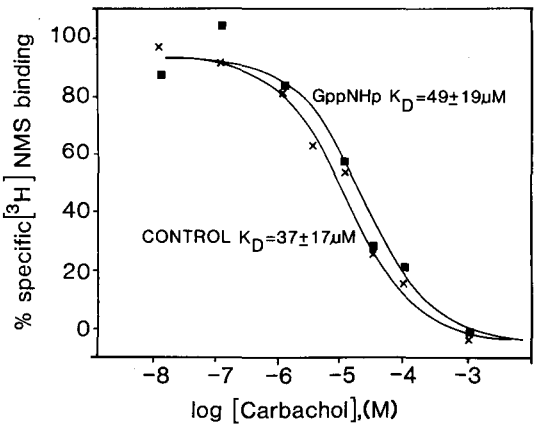

Fig 2. Displacement of [^{3}H]NMS by the agonist carbachol in the absence or presence of 0.1 mM of the hydrolysis-resistant GTP analog 5'-guanylylimidodiphosphate (GppNHp) in human gastric mucosa.

equally high affinity for all known subtypes of muscarinic receptors (7). As expected, the displacement curve of unlabeled vs labeled NMS was steep and corresponded to a single binding site (Figure 1). The affinity of NMS was K_D = 0.39 ± 0.08 nM (n = 11) and the binding capacity was 27.7 ± 5.6 fmol/mg protein (n = 11). There was no statistically significant difference between tissue obtained from gastrectomies because of carcinomas (n = 7, K_D = 0.35 ± 0.11 nM, binding capacity 26.6 ± 5.3 fmol/mg protein) or antrectomies because of ulcer disease (n = 4, K_D = 0.44 ± 0.11 nM, binding capacity 27.7 ± 5.6 fmol/mg protein).

Pirenzepine was employed to assess whether M1 receptor subtypes were present. This M1 antagonist displayed NMS in a steep curve (Figure 1), which was best represented by a single binding site. The binding affinity of pirenzepine was K_D = 148 ± 32 nM (n = 13) and did not differ between tissues obtained from gastrectomies because of carcinomas (n = 8, K_D = 117 ± 29 nM, binding capacity 28.2 ± 4.9 fmol/mg protein) or benign diseases (n = 5, K_D = 198 ± 75 nM, binding capacity 27.3 ± 10.8 fmol/mg protein). These results suggested the presence of non-M_1 receptors.

Previous experiments had shown that M3 receptors present in porcine gastric mucosa had a low affinity for carbachol of about 20 μM (22). In contrast, cardiac or smooth muscle muscarinic receptors had a major portion of high-affinity binding sites for carbachol with K_D values of 100–300 nM. In addition, the mucosal subtype did not show a decrease in agonist affinity in the presence of guanine nucleotides, while these nucleotides significantly decreased agonist binding affinity in cardiac and smooth muscle homogenates containing predominantly M2 receptors (22, 31, 32). We therefore investigated the properties of human mucosal receptors using displacement experiments of the agonist carbachol against tritiated NMS in the presence or absence of GppNHp, a hydrolysis-resistant analog of the natural guanine nucleotide, GTP. The binding affinity of carbachol was 37 ± 17 μM in the absence and 48 ± 19 μM (n = 6) in the presence of 0.1 mM of GppNHp, which does not represent a statistically significant decrease (Figure 2). The low affinity of carbachol was similar to the one observed in porcine mucosa (22) or rat gastric parietal cells (26).

A further classification of the muscarinic receptor type was achieved by using M2 (AF-DX 116) and M3 receptor (HHSiD and SiHC) specific antagonists. Each compound displaced NMS completely with a steep slope suggestive of a single binding component (Figure 3). The affinity of the M3 antagonists HHSiD and SiHC was (K_D) 78 ± 23 nM (n = 5) and 5.6 ± 1.8 nM (n = 3), respectively, and that of the M2 antagonist AF-DX was (K_D) 4043 ± 1011 nM (n = 3). There was no evidence for a second binding site using either displacer.

DISCUSSION

The study classifies muscarinic receptors in human gastric mucosa by use of selective muscarinic antagonists. Pirenzepine, the classical M1 antago-

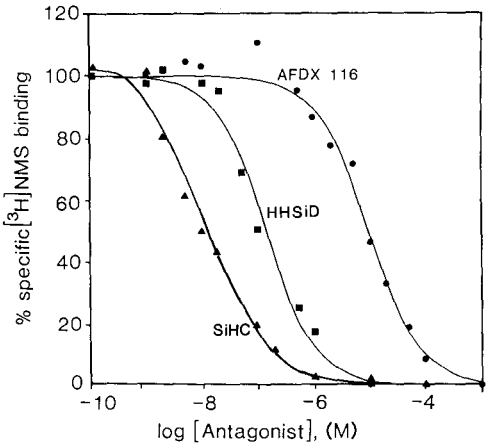

Fig 3. Displacement of [^{3}H]NMS by the selective antagonists hexahydrosiladifenidol (HHSiD), silahexocyclium (SiHC), and AF-DX 116 in human gastric mucosa.

nist, had a low affinity of less than 100 nM for mucosal receptors, which provides no evidence for the M1 subtype. As the affinity of AF-DX 116 was rather low, the presence of cardiac M2 subtypes also can be largely excluded.

The M3 antagonist HHSiD had an affinity of 78 nM for the mucosal receptors. This is at the lower end of the range reported for the affinity of HHSiD to M3 receptors in glandular tissues (23, 24, 36). The affinity of HHSiD was lower than that observed previously in rat gastric parietal cell preparations or porcine mucosa, which was between 3 and 6 nM (22, 26). It therefore appears that species differences in the affinity of HHSiD to M3 receptors exist. This is supported by a study of rabbit parietal cells in which the affinity of HHSiD was between 79 and 211 nM as determined by functional or binding assay, respectively (30). Experiments with silahexocyclium (SiHC) also showed a high affinity of this M3 antagonist, which was 6 nM and thus comparable to the affinity of 3 nM K_D observed in rat gastric parietal cells (26). The very low affinity of AF-DX 116 further supports the occurrence of M3 receptors. The pattern of ligand affinities would also fit the M5 receptor recently described by Bonner and coworkers (4). However, the mRNA coding for this receptor was not found in peripheral tissues (4), and therefore it seems unlikely that M5 receptors are present in gastric mucosa. However, our technique does not permit us to exclude entirely this possibility or the presence of a small population of a different subtype of muscarinic receptors.

In previous experiments carbachol was observed to possess a low affinity to rat, dog, or porcine gastric mucosal muscarinic receptors (22, 29, 37, 38). In contrast to muscarinic receptors in heart and gastric smooth muscle, which display a large decrease in high-affinity binding sites in the presence of guanine nucleotides, gastric mucosal receptors were not susceptible to modulation by guanine nucleotides (22, 38). Similar properties were found in human gastric mucosa in this study. In control experiments guanine nucleotides significantly decreased the agonist binding affinity in smooth muscle (39), which corresponds to observations made in porcine and canine gastric smooth muscle (22, 38).

In summary, human gastric mucosa was found to possess muscarinic receptors that may be classified as belonging to the M3 subtype. This corresponds to the HM4 receptor (5, 6) or the m3 (3, 4, 7) and mAChR III (1, 2, 8) as classified by the various nomenclatures (9) of the cloned muscarinic receptors. A major portion of mucosal mucarinic receptors is located on parietal cells (29). It therefore seems likely that muscarinic stimulation of acid secretion involves M3 receptors in humans, although studies using isolated human parietal cells are needed to establish this proposal definitely.

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