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MUSCARINIC RECEPTORS IN ISOLATED GASTRIC FUNDIC MUCOSAL CELLS

BINDING-ACTIVITY RELATIONSHIPS

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Abstract—Muscarinic receptors are involved in the control of gastric acid secretion. The characteristics of (^{3}H)-*N*-methyl-scopolamine [(^{3}H)-NMS] binding to isolated cells from rabbit fundic gastric mucosa and inhibition of this binding by muscarinic agonists, antagonists and other pharmacological agents known to regulate acid secretion are reported. Specific binding for (^{3}H)-NMS was described: antagonists interact with high affinity sites (K_D = 0.5 nM) whereas binding curves for agonists clearly deviated from the simple mass action isotherm with a flattening of the curve suggesting the presence of more than one class of sites. The low affinity sites for agonists are in the micromolar range. Pirenzine, a gastroselective antimuscarinic compound, known to differentiate between M1 and M2 sites, inhibited (^{3}H)-NMS binding with an IC_{50} of 0.05 μM.

On the same gastric cell population, muscarinic agonist carbachol stimulated (^{14}C)-aminopyrine accumulation in a dose-dependent manner with an ED-50 of 10 μM, value close to that needed to 50% inhibit (^{3}H)-NMS binding. This stimulation was competitively inhibited by muscarinic antagonists and pA_2-values for atropine, QNB and pirenzepine, calculated from linear Schild plots, were in the following order: 9.2 for atropine, 8.6 for QNB and 7.0 for pirenzepine.

In conclusion, fundic gastric mucosal cells from rabbit, isolated with collagenase and EDTA, contained specific muscarinic receptors coupled to the acid secretory mechanism and pirenzepine interact with these receptors with an intermediate affinity suggesting the presence of functional M2-sites.

Acid and pepsinogen secretions from mammalian gastric mucosa are increased by vagal stimulation. This observation justified the anticholinergic therapy in the treatment of peptic ulcer.

Muscarinic receptors have been characterized in both smooth muscles [1] and mucosa [2] of the stomach wall, but the intracellular mechanism of this cholinergic control is not well known and the use of dispersed gastric glands or cells from fundic mucosa of various species, devoid of central and peripheral controls, was proposed to clarify it. Isolated gastric parietal cells from rabbit, guinea-pig or dog were shown to be sensitive to gastrin, histamine and carbachol [3, 4]: the stimulating effect of these secretagogues on aminopyrine accumulation (an index of acid secretion from isolated gastric cells or glands) and oxygen consumption, suggested that parietal cells is controlled by three receptors for histamine, acetylcholine and gastrin. Successively, histamine [5] and gastrin [6] receptors were characterized by using ligand-binding methods. The presence of cholinergic binding sites for (^{3}H)-quinuclidyl-benzilate or (^{3}H)-*N*-methyl-scopolamine was also demonstrated in both isolated broken cells [7] and in isolated intact cells [8].

In the present paper, we report the characteristics of the muscarinic receptor in an isolated gastric fundic mucosal cell preparation from rabbit, using (^{3}H)-*N*-methyl-scopolamine as ligand. We also present binding characteristics of pirenzepine, a new gastro-selective anticholinergic compound used as an antisecretory drug [9] which was presented as able to differentiate between M1 and M2 muscarinic receptor sites [10]. These isolated cells accumulate the weak base aminopyrine in their tubulo-vesicular system in response to carbachol, histamine and gastrin stimulations [11, 12]. In a last part, by using this aminopyrine accumulation method, we report relationships between muscarinic receptor occupancy by agonists and antagonists and biological event (acid secretion).

MATERIALS AND METHODS

Pirenzepine was a gift from Boehringer–Ingelheim (France). Acetylcholine, carbachol, pilocarpine, oxotremorine, scopolamine, histamine, atropine, HEPES and bovine serum albumin (fraction V) (BSA) were from Sigma Chemical Co. (St Louis, MO). Quinuclidyl benzilate (QNB), (^{3}H)-*N*-methyl-scopolamine (80 Ci/mmole) and dimethylamine-(^{14}C)-aminopyrine (118 mCi/mmole) were from Amersham (U.K). Earle's balanced salt solution was from Biomerieux (France) and collagenase (0.8 U/mg) was from Serva (Heidelberg, West Germany).

Medium A: 132 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM HEPES, 0.2% glucose, 0.2% BSA, 0.2% phenol red, pH 7.4.

Medium B: Earle's balanced salt solution without bicarbonate containing 10 mM HEPES and 0.2% BSA, pH 7.4.

Preparation of isolated fundic gastric mucosal cells. Cell isolation was carried out following the

collagenase/EDTA procedure as described previously [11]. Fundic mucosa was scraped and tissue fragments were chopped in small cubes, then dispersed in medium A (gassed with 95% O_2–5% CO_2) containing 0.3 mg/ml collagenase. After 15 min incubation at 37°, tissue fragments were allowed to settle and the medium was discarded. The fragments were washed in Ca^{2+}–Mg^{2+}-free medium A containing 2 mM EDTA and then incubated in this medium for 10 min. Fragments were transferred into medium A containing fresh collagenase (0.3 mg/ml) and incubated for 30 min at 37° under continuous gassing (O_2/CO_2). Cell suspension was centrifuged for 5 min at 200 *g*, then washed twice with medium A. This procedure gave about 3×10^7 cells per g of wet mucosa with 95% viability (trypan blue exclusion). The mixed cell population contained 45% parietal cells, as determined by electron microscopy.

Aminopyrine accumulation. Cell suspension in Earle's medium B (1.5×10^6 cells per ml, 1 ml) was incubated with 0.05 μCi of (^{14}C)-aminopyrine (3 μM) with or without stimulants and inhibitors at 37° for 20 min under continuous gassing (O_2/CO_2) (final volume: 1.5 ml). Triplicate samples (0.4 ml of each tube) were layered over 0.9 ml ice-cold medium B and centrifuged for 1 min (microfuge Eppendorf). The cell pellet was suspended in 0.1 ml 10% $HClO_4$ and the radioactivity was measured in a β liquid scintillation counter (Beckman LS 7500). AP accumulation represents the percent of the radioactivity associated with cells.

Binding studies. Binding experiments were performed in duplicate with isolated cells suspended in Earle's medium B. (a) Association: (^{3}H)-NMS (6.5×10^{-10} M) was added to the cell suspension (usually 5×10^6 cells per ml) and incubated for various temperatures (4°, 25°, 37°). At specified times, 0.2 ml of the suspension was removed, diluted with 0.6 ice-cold medium B and centrifuged in a microfuge. The supernatant was discarded and 0.1 ml 10% $HClO_4$ was added to the cell pellet. The radioactivity of the pellet was measured in 8 ml ACS (Amersham) in a liquid scintillation counter. Non-specific binding was determined in parallel by incubating cells with (^{3}H)-NMS in the presence of an excess of unlabelled NMS (5 μM). Specific binding (bound tracer) corresponds to the difference between total binding [(^{3}H)-NMS alone] and non-specific binding [(^{3}H)-NMS plus excess of unlabelled NMS]. The free tracer concentrations were calculated from the differences between total and bound (^{3}H)-NMS concentrations in the incubation medium. (b) Dissociation: after a 20-min incubation period at 37° in the presence of (^{3}H)-NMS alone or (^{3}H)-NMS plus unlabelled NMS, cells were centrifuged at 200 *g* for 2 min. The supernatant was discarded and the cell pellet resuspended in the same volume of fresh medium B. Then, the cell suspension was 50-times diluted with fresh medium B with or without 1 μM unlabelled NMS. At specified times, duplicate samples of 10 ml suspensions were centrifuged for 5 min. The radioactivity associated with the cell pellet was measured as described for association. (c) Saturation analysis: various amounts of (^{3}H)-NMS (0.25–4 nM) were incubated with cells for 20 min at 37° in the presence or in the absence of unlabelled NMS (final volume: 0.2 ml). Specific binding was determined as for association. (d) Competition studies: the inhibition of (^{3}H)-NMS binding by unlabelled muscarinic agonists and antagonists as well as non-cholinergic substances was determined by incubating cells for 20 min with (^{3}H)-NMS (0.5 nM) and various concentrations of these compounds. (^{3}H)-NMS specific binding was determined as for association.

RESULTS

Specific (^{3}H)-NMS binding as a function of time

Specific binding of (^{3}H)-NMS to gastric mucosal cells at 37° was maximal by 10 min incubation. When reducing incubation temperature from 37° to 25° and 4°, the time needed to reach equilibrium dramatically increased (Fig. 1). In these experiments, non-specific binding was 16% of total binding.

(^{3}H)-NMS specific binding increased linearly with cell concentrations up to 8×10^6 cells per ml (Fig. 2). We used 5×10^6 cells per ml in the following studies.

Dissociation

Figure 3 shows the dissociation curves after a 50-fold dilution of the incubation medium with medium B alone or medium B plus 1 μM unlabelled NMS. 70% of the specific bound radioactivity was dissociated by 70 min with a $t_{\frac{1}{2}}$ of 28 min. The extent of NMS dissociation significantly increased to 80% during the same period in the presence of 1 μM unlabelled NMS. This increase could be interpreted as an interaction between binding sites.

Saturation of binding sites

Various concentrations of (^{3}H)-NMS (0.25–4 nM) with or without unlabelled NMS (5 μM) were incubated with gastric mucosal cells for 20 min at 37°.

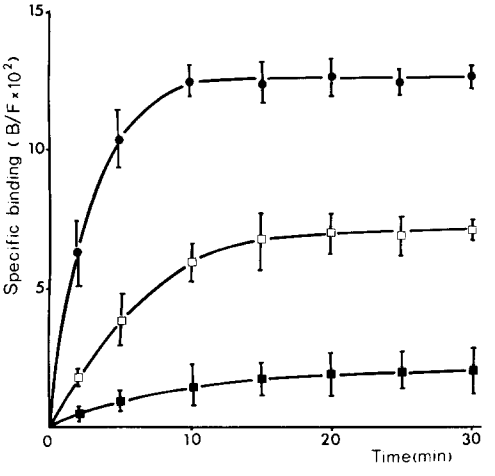

Fig. 1. (^{3}H)-NMS specific binding to isolated gastric mucosal cells as a function of time. (^{3}H)-NMS (0.65 nM) was incubated with cells (5×10^6 per ml) in the absence (total binding) or in the presence (non-specific binding) of 5 μM unlabelled NMS, then ligand binding was measured. Specific binding (difference between total and non-specific binding) was expressed as bound over free ratio (B/F) and plotted as a function of time. Each value is the mean of triplicate determinations. ● + 37°, □ + 25°, ■ + 4°.

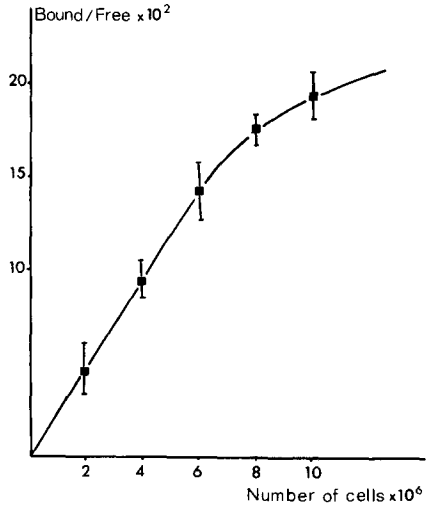

Fig. 2. Relationship between (^{3}H)-NMS specific binding and cell concentration. (^{3}H)-NMS (0.65 nM) was incubated with various cell concentrations (2–10 × 10^6 cells per ml) for 20 min at 37°. Non-specific binding was determined for each cell concentration by adding 5 μM unlabelled NMS to the incubation medium. Specific B/F ratios were plotted as a function of cell concentration.

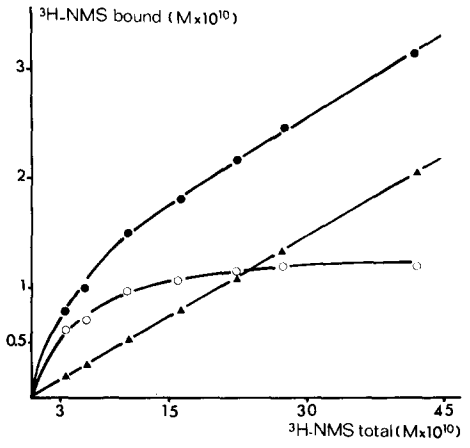

Fig. 4. Association of (^{3}H)-NMS with gastric cells at steady state. Various concentrations of (^{3}H)-NMS (0.25–4 nM) with (▲——▲) or without (●——●) 5 μM unlabelled NMS were incubated with cells for 20 min at 37°. Binding was expressed as bound labelled ligand as a function of (^{3}H)-NMS concentrations. (○——○) Specific binding is the difference between total and non-specific binding. Values are means of six separate experiments.

As shown in Fig. 4, non-specific binding increases linearly with ligand concentration and the specific binding appears to be a saturable process. Scatchard's plot is linear, showing a single class of high affinity binding sites and the total number of binding sites calculated from this representation was 15000 sites per cell (Fig. 5). A statistical analysis of the dissociation constants (K_D) determined from six separate experiments gave the following value:

$$K_D = 0.46 \pm 0.05 \text{ nM (mean} \pm \text{S.D.)}.$$

Hill coefficient from those data was not statistically different from unity, compatible with the simple mass-action isotherm of Langmuir for the association model $R + L \rightleftharpoons RL(k)$,

$$nH = 0.87 \pm 0.12.$$

This K_D-value is exactly the same than that obtained by Albinus [8] in guinea-pig cells and in good agreement with those from a variety of rat organs as shown by Hammer [14].

When using unlabelled QNB for the determination of the non-specific binding and (^{3}H)-NMS as tracer, a similar saturation curve was obtained with a K_D = 0.4 nM.

Competitive determination of affinity constants

(^{3}H)-NMS binding to isolated gastric mucosal cells was competitively inhibited by increasing concentrations of unlabelled NMS (Fig. 6). IC_{50} value was 1 ± 0.5 nM (n = 5).

The pharmacological specificity of the receptor was characterized by studying the effects of various muscarinic agonists and antagonists as well as other

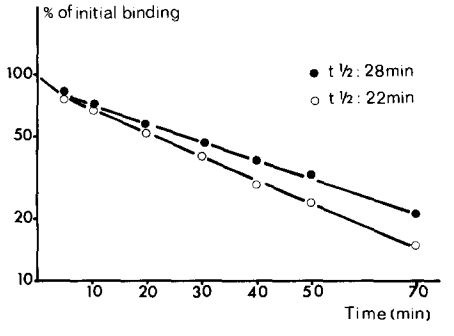

Fig. 3. Dissociation of (^{3}H)-NMS specific binding to gastric mucosal cells. (^{3}H)-NMS specifically remaining associated with cells after a 50-fold dilution of the incubation medium was expressed as the percent of initial ligand binding and plotted as a function of time elapsed after the dilution. ● 50 × dilution alone, ○ 50 × dilution plus 1 μM unlabelled NMS.

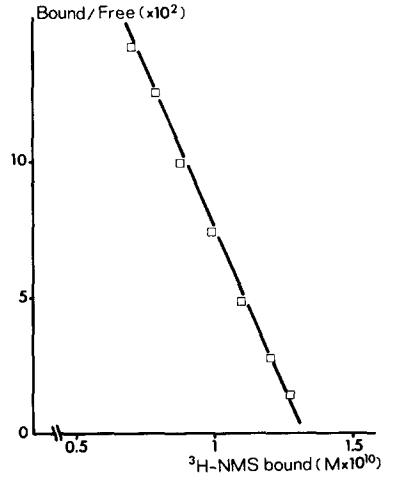

Fig. 5. Scatchard's plot representation of the data from the experiments shown in Fig. 4.

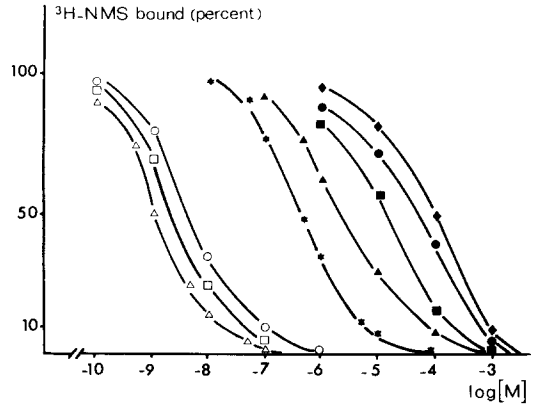

Fig. 6. Dose–response curves of muscarinic agonists and antagonists on (^{3}H)-NMS binding to isolated gastric cells. (^{3}H)-NMS (0.5 nM) was incubated with cells for 20 min at 37° in the presence of varying concentrations of unlabelled compounds. Results were expressed as the percent of maximal specific binding ($B/B_o \times 100$) and plotted against concentrations of the respective compounds. Results are means of 3–6 separate experiments. *N*-methyl-scopolamine (△——△), atropine (□——□), QNB (○——○), pirenzepine (★——★), oxotremorine (■——■), pilocarpine (▲——▲), carbachol (●——●), acetylcholine (◆——◆).

compounds known to interfere in the acid secretory process such as histamine, gastrin, somatostatin and cholecystokinin and other peptides like Met-enkephalin:

(a) muscarinic antagonists (QNB, atropine, *N*-methyl-scopolamine) inhibited (^{3}H)-NMS binding with IC_{50} in the nanomolar range, while agonists (oxotremorine, acetylcholine and carbachol) and the partial agonist pilocarpine inhibited (^{3}H)-NMS binding at higher concentration (between 3 and 100 μM). This (^{3}H)-NMS binding was also competitively inhibited by pirenzepine with an IC_{50} of 0.5 μM (100 × higher than for atropine) (Fig. 6 and Table 1). Hill coefficients (*nH*-values, Table 1), calculated by linear regression and analysed for statistically significant deviation from unity, were statistically

Table 1. Abilities of various agonists and antagonists of the muscarinic receptor to inhibit (^{3}H)-NMS binding to isolated gastric mucosal cells

	IC_{50} (mol/l)	Hill (*nH*) coefficient
Antagonists		
N-methyl-scopolamine	1×10^{-9}	0.88
Atropine	3×10^{-9}	0.83
QNB	6×10^{-9}	0.78
Pirenzepine	5×10^{-7}	0.87
Agonists		
Oxotremorine	3×10^{-6}	0.68*
Pilocarpine	2.5×10^{-5}	0.63*
Carbachol	7.5×10^{-5}	0.58*
Acetylcholine	1×10^{-4}	0.57*

IC_{50} represents the concentration of drug causing 50% inhibition of specific binding. *nH* (Hill coefficient) was determined from displacement curves. * are values significantly different from unity with a curvilinear Hill plot.

Table 2. Abilities of various pharmacological agents to inhibit (^{3}H)-NMS binding to isolated gastric mucosal cells

Pharmacological agent	$B/Bo \times 100$
Gastrin (HG-17) (1×10^{-6} M)	90
Histamine (1×10^{-4} M)	94
Met-enkephalin (1×10^{-6} M)	83
Somatostatin (5×10^{-7} M)	85
CCK-OP (1×10^{-5} M)	97
Nicotine (1×10^{-5} M)	30*
Hexamethonium (1×10^{-3} M)	35*

Results were expressed as the percent of maximal specific binding at equilibrium for indicated concentrations of the various agents. Standard deviations from B/Bo determinations were less than 25%. * are values significantly different from controls.

different from 1.0 for acetylcholine, carbachol, oxotremorine and pilocarpine. This suggested either the existence of some interaction between binding sites or the presence of two categories of sites with high and low affinity for agonists.

(b) nicotine and hexamethonium (a nicotinic ganglioplegic blocker) at high concentrations inhibited (^{3}H)-NMS binding, while histamine, gastrin, CCK-7, somatostatin and met-enkephalin were without significant inhibitory effect (Table 2).

(^{14}C)-*Aminopyrine accumulation*

(a) Carbachol-induced AP accumulation into parietal cells was a concentration-dependent process in the range 1×10^{-7}–1×10^{-4} M with a maximal response for 1×10^{-4} M carbachol concentration. Atropine caused a rightward shift of the dose–response curve for carbachol proportionally to the

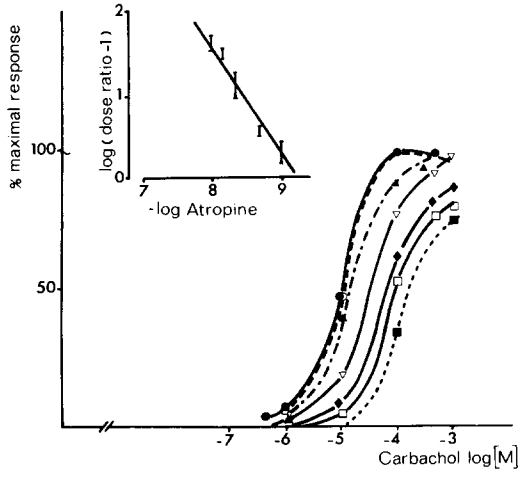

Fig. 7. Dose–response curves of (^{14}C)-aminopyrine accumulation by isolated cells stimulated by carbachol (●) in the presence of different concentrations of atropine. Cells were incubated for 20 min at 37° with 0.05 μCi of (^{14}C)-aminopyrine, with various concentrations of carbachol and indicated concentrations of atropine: 1×10^{-8} M ■; 7.5×10^{-9} M ◆; 5×10^{-9} M ◆; 2.5×10^{-9} M △; 1×10^{-9} M ▲; 1×10^{-10} M ○. The inset represents a plot of log (dose ratio −1) vs −log (atropine concentrations) (Schild's plot).

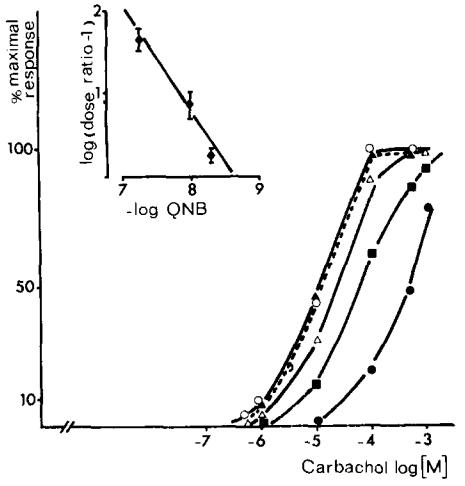

Fig. 8. Dose–response curves of (^{14}C)-aminopyrine accumulation by isolated cells stimulated by carbachol (▲) in the presence of different concentrations of QNB. See legend Fig. 7. 5×10^{-8} M ●; 1×10^{-8} M ■; 5×10^{-9} M △; 1×10^{-9} ○. The inset represents the Schild's plot from these data.

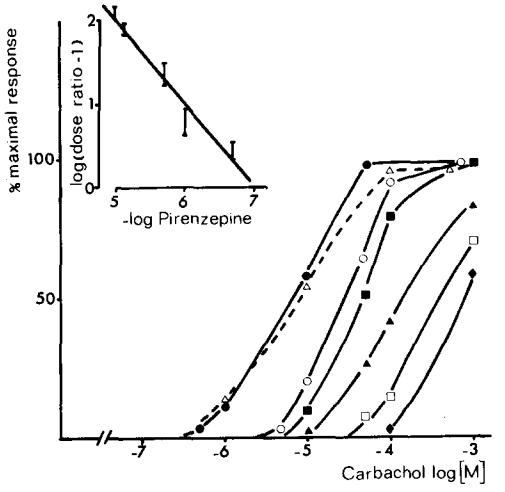

Fig. 9. Dose–response curves of (^{14}C)-aminopyrine accumulation by isolated cells stimulated by carbachol (●) in the presence of different concentrations of pirenzepine. See legend Fig. 7. 1×10^{-5} M ◆; 7.5×10^{-6} M □; 2.5×10^{-6} M ▲; 1×10^{-6} M ■; 5×10^{-7} M △. The inset represents the Schild's plot from these data.

antagonist concentrations (Fig. 7). The same was observed with QNB (Fig. 8) and with pirenzepine (Fig. 9). The calculated pA_2-values for the three antagonists were respectively 9.2 for atropine, 8.6 for QNB and 7.0 for pirenzepine. The slopes of the Schild's plots were not statistically different from unity indicating that, in this model, the antagonism for atropine, QNB and pirenzepine appeared to be of a competitive nature. Moreover, the IC_{50} values, calculated from data shown in Fig. 7, 8 and 9 (inhibition of carbachol-induced AP accumulation) are closely related to that obtained from the competition binding curves (Fig. 6):

	Binding	*Aminopyrine*
Atropine	3×10^{-9} M	5×10^{-9} M
QNB	6×10^{-9} M	15×10^{-9} M
Pirenzepine	0.5×10^{-6} M	1×10^{-6} M

(b) IBMX (10 μM) strongly increased AP accumulation, but the maximal response to carbachol was not potentiated by the presence of the phosphodiesterase inhibitor (Table 3). The withdrawal of extracellular Ca^{2+} in the presence of 2 mM EGTA in the incubation medium dramatically reduced AP accumulation (Table 3). This is in agreement with the stimulating effect of the influx of Ca^{2+} into parietal cell during the H^+ secretory process.

DISCUSSION

This paper evidenced a close relationship between the acid secretory response to carbachol and the occupancy of the muscarinic receptor in the same fundic gastric mucosal cell preparation.

This cell population, isolated from rabbit fundic mucosa, contains high-affinity receptors for a classical labelled muscarinic antagonist, (^{3}H)-*N*-methylscopolamine used as ligand because of its low non-specific binding to cells.

In our experimental conditions, binding was time and cell-concentration dependent. Equilibrium was achieved by 10 min at 37° and the half-time of dissociation (28 min) was similar to that observed in brain with (^{3}H)-QNB [13]. The increase in the dissociation rate in the presence of unlabelled NMS could be interpreted as an interaction between binding sites. Scatchard's analysis from saturation experiments indicated the presence of only one high-affinity category of sites and dissociation constant (0.46 nM) and Hill coefficient ($nH = 0.87$) were in agreement with previous observation in this [7, 8] and other tissues [2]. The receptor is a protein: specific binding disappeared when cells were treated with pronase.

The receptor studies are specific for cholinergic drugs; other pharmacological agents known to mod-

Table 3. Effects of IBMX and Ca^{2+} on carbachol-induced aminopyrine accumulation in isolated gastric mucosal cells

	Aminopyrine accumulation (%)		
	No addition	+ IBMX (10 μM)	+ EGTA (2 mM)
Control cells (n = 5)	8.1 ± 0.8	22.6 ± 2.8	6.41 ± 1.4
Carbachol (10 μM) (n = 5)	14.6 ± 1.8	28.0 ± 3.1(*)	7.2 ± 1.2

Values are means ± S.D. n is the number of typical experiments. (*) The observed value for IBMX + carbachol was the same as the sum of the values from separated components.

ify gastric acid secretion did not affect (^{3}H)-NMS binding such as gastrin, CCK, somatostatin and histamine.

As it was observed for smooth muscles [2], heart [14] and brain [15], muscarinic antagonists compete with (^{3}H)-NMS high-affinity binding sites without significant differences between them. The parallel inhibition of binding observed with agonists was in the low affinity range. The existence of these two statistically different subclasses of binding sites are in agreement with the *H* and *L* muscarinic sites described by Birdsall [16].

As opposed to the results from Hammer [2] and Albinus [8], our competitive experiments on the isolated cells with pirenzepine showed that muscarinic receptors coupled to the acid secretory process are homogeneous (Hill coefficient for pirenzepine = 0.87) and with low affinity (IC_{50} 0.5 μM). The competition binding curve for pirenzepine was statistically different from the high-affinity domain for antagonists and from the low affinity domain for agonists.

Carbachol stimulates aminopyrine accumulation in a dose-dependent manner. The addition of 10 μM IBMX to carbachol enhanced this stimulation but without potentiation between the two compounds; a strong potentiation was observed when IBMX was added to histamine stimulation [18]. The effect of carbachol was highly dependent from the presence of Ca^{2+} in the incubation medium, as previously shown by Berglindh [3] and Soll [4].

If we compare binding data and AP accumulation data in isolated cells, a close relationship was shown with the different agonists and antagonists studied. The dose–response curve to carbachol presented a maximum for high carbachol concentrations (1×10^{-4} M) suggesting that low affinity sites are involved for agonists in agreement with binding data. Atropine, QNB and pirenzepine induced a rightward shift of the dose–response curve to carbachol indicating that these agents are competitive antagonists for acid secretion from parietal cells. Schild's plots were linear and the pA_2-values (9.2 for atropine, 8.6 for QNB) are in agreement with a mediation of acid secretion by high-affinity sites for antagonists on parietal cells, while pA_2-value for pirenzepine suggested that its inhibitory effect on acid secretion was mediated by only one class of low affinity muscarinic sites. So, in these experiments, pirenzepine was not able to evidence M1-sites in gastric mucosa. This conclusion agrees with the respective localization of these sites (only M2-sites described in gastric mucosa). However, one cannot exclude that M1-sites, if present in this tissue and associated to the acid secretory process, had been destroyed during the collagenase digestion.

The use of other selective antimuscarinic agents beside pirenzepine together with this model of isolated cells from gastric mucosa can help to elucidate the cholinergic control of gastric acid secretion.

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