Biochemical Pharmacology, Vol. 36, No. 18, pp. 2957–2961, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

CHARACTERIZATION OF THE MUSCARINIC RECEPTOR SUBTYPE IN ISOLATED GASTRIC FUNDIC CELLS OF THE RABBIT

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(*Received* 12 *August* 1986; *accepted* 23 *March* 1987)

Abstract—The characteristics of the muscarinic receptor in isolated gastric fundic cells from rabbit were determined by radioligand binding techniques and functional tests. The dissociation constants (K_Ds) of selective (hexahydrosiladifenidol and pirenzepine) and non-selective (*N*-methylscopolamine and atropine) muscarinic receptor antagonists obtained in competition experiments vs [^{3}H]-*N*-methylscopolamine were compared with the pA_2 values of the drugs as inhibitors of carbachol-stimulated [^{14}C]-aminopyrine accumulation (an index of acid secretion) in the gastric fundic cells. Good correlations were found between the ability of the drugs to inhibit acid secretion and their affinity for muscarinic receptors in the gastric fundic cells. The rank order of potency in both tests was *N*-methylscopolamine > atropine > hexahydrosiladifenidol > pirenzepine. The character of the muscarinic receptor subtype present on gastric fundic cells was established by comparing the affinity values of the compounds for this receptor with those for the receptors in other rabbit tissues. It was found that only pirenzepine and hexahydrosiladifenidol displayed tissue selectivity in their binding profiles. The K_Ds for pirenzepine were 13 nM for the M_1 receptor of the cerebral cortex and about 500 nM for the M_2 receptors of the submandibular and gastric glands and heart. Differently from pirenzepine, hexahydrosiladifenidol showed about 10-fold discrimination between the M_2 subtype of the gland (K_D = 31 nM) and the M_2 subtype of the heart (K_D = 330 nM).

During the last five years, growing evidence in favor of the existence of different subclasses of muscarinic receptors has accumulated [1]. Pirenzepine (PZ) was the first muscarinic antagonist to show heterogeneity in binding studies [2], a property that correlated with the pharmacological activity of the drug [3–5]. These characteristics have largely contributed to the subclassification of muscarinic receptors in M_1 (high affinity for PZ) and M_2 (low affinity for PZ). Recently, a novel compound, hexahydrosiladifenidol (HHSiD), has been described which is able to differentiate between subtypes of M_2 receptors in smooth muscle and heart on the basis of pharmacological tests [6].

The objective of this study was to characterize the muscarinic receptor subtype on gastric fundic cells (GFC) which play a role in the regulation of acid secretion. For this purpose we performed parallel studies utilizing both non-selective, *N*-methylscopolamine (NMS) and atropine (ATR), and discriminating (PZ, HHSiD) compounds and comparing their binding affinities with their ability to inhibit carbachol-stimulated [^{14}C]aminopyrine (AP) accumulation.

MATERIALS AND METHODS

Reagents and media. Drugs, radioligands and enzymes were obtained from the following sources: [^{3}H]-NMS (84.8 Ci/mmol), [^{3}H]-PZ (84 Ci/mmol), [^{14}C]-AP (108 mCi/mmol) purchased from NEN Boston, MA, U.S.A.; NMS and ATR purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. PZ and 3-quinuclidinyl benzilate racemic mixture (QNB) was synthesized at Dr. K. Thomae, GmbH (Biberach, F.R.G.). HHSiD was generously supplied by Dr. E. Mutschler (Dept. of Pharmacology, University of Frankfurt, Frankfurt, F.R.G.); collagenase from *Clostridium histolyticum* (0.6–0.8 U/mg) was purchased from Serva Feinbiochemica GmbH, (Heidelberg, F.R.G.).

Earle's balanced salt solution was from Biomerieux (France) and Hank's balanced salt solution was from Flow Laboratories (Italy).

Solutions. 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: Medium A containing 2 mM EDTA, without Ca^{2+} and Mg^{2+}. Media A and B were additionned with 50 μg/ml streptomycin and 100 U/ml penicillin.

Medium C: Medium A without antibiotics. Na^+/Mg^{2+} HEPES buffer: 100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES, pH 7.4.

Isolation of gastric fundic cells. Male New Zealand albino rabbits, 2–2.5 kg (Pellizzari, Bergamo, Italy) were used. Fed rabbits were killed by a blow on the head and bled. The stomach was quickly excised, opened and washed in phosphate buffered saline (PBS) at 4°. The mucosa of the oxyntic gland portion was cleaned with tissue paper, scraped off with the edge of a glass slide and then cut into cubes of 200 μm thickness with a McIlwain mechanical tissue chopper at top blade force. Abouth 9 g of mucosa were obtained from one rabbit. Gastric fundic cells were then obtained following minor modifications [7] of

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the protocol described by Soll [8]. Briefly, the cubes were added to 18 ml of medium A containing 0.3 mg/ml collagenase in a 50 ml celstir flask gassed with 95% O_2–5% CO_2, and incubated for 15 min at 37° with shaking. At the end of the incubation the mucosal fragments were allowed to settle and the medium discarded. The fragments were rinsed twice with medium B and incubated in fresh medium B for 10 min. After settling, the mucosal fragments were rinsed twice with medium A and then incubated in fresh medium A as described earlier. At the end of this incubation the cell suspension was lightly centrifuged for 2 min at 50 *g* and the supernatant was discarded. The pellet was resuspended in the same volume of medium A containing fresh collagenase and incubated again for 20–30 min, during which time the remaining tissue fragments were broken up by repetitive aspiration/expulsion through a Pasteur pipette.

The cell suspension thus obtained was diluted with an equal volume of medium A and filtered through a 240 μm Nylon mesh; the filtrate was spun at 200 *g* for 5 min in 50 ml centrifuge tubes; the supernatant was aspirated and discarded. The cells were resuspended in the initial volume of medium C and centrifuged again at 200 *g* for 5 min. After a final resuspension in medium C the cells were passed through a 62 μm nylon mesh and spun as above. The final pellet was resuspended in 15 ml of Earle's medium and the cells were counted in a Thoma cell.

A standard preparation contained 20–30 × 10^6 cells/g wet tissue, about 50% of which were parietal cells. Cell viability, determined by trypan blue exclusion, was 80–90%.

Determination of ^{14}C*-aminopyrine accumulation.* Cells were suspended in Earle's medium (0.6 × 10^6 cells/ml). Two ml of the cell suspension were incubated with 1 μM[^{14}C]-AP and increasing concentrations of carbachol with and without antagonist in an atmosphere of 95% O_2–5% CO_2. Incubation was carried out in a 24-well tissue culture cluster at 37° for 20 min. Triplicate aliquots (0.5 ml) of each sample were layered over 1 ml of ice-cold Hank's balanced salt solution in 1.5 ml microfuge tubes and centrifuged at 12,000 rpm for 3 min using an Eppendorf microcentrifuge. At the end of the centrifugation, 200 μl aliquots of the supernatant were added to 5 ml plastic scintillation vials containing 4 ml Instagel (Packard) and counted for radioactivity. The pellets were carefully washed three times with 1.5 ml saline and then left to dry. The tips of the microcentrifuge tubes containing the pellets were cut off and 200 μl of tissue solubilizer (Lumasolve, Lumac) were added and left to stand overnight. Samples were then counted for radioactivity after addition of 4 ml of liquid scintillation medium (toluene: dimilume 10 : 1 v/v, Packard).

Binding studies with gastric fundic cells. Binding experiments were performed with some technical modifications of the method previously described [7]. The cell suspension (5 × 10^6 cells/ml) was incubated for 45 min at 30° with 0.4 nM[^{3}H]-NMS and various concentrations of the cold ligand (final volume 200 μl). The incubation was terminated by adding 5 ml of ice-cold Tris–HCl buffer (pH 7.5) and the cell suspension was filtered under reduced pressure through glass fiber filters (GF/B Whatman). The filters were washed twice with 5 ml of ice-cold Tris buffer, pH 7.5, and then introduced into 5 ml plastic scintillation vials. 4 ml of Filter-count (Packard) were then added and the radioactivity was determined by liquid scintillation counting. Specific binding was defined as that displaceable by 1 μM QNB or NMS and averaged to more than 80% of the total binding.

Binding studies in tissue homogenates. Fed rabbits were killed by a blow on the head and bled. Submandibular glands were removed, cleaned and homogenized with an Ultra-Turrax at maximal speed for 30 sec followed by a Potter–Elvehjem (15 strokes) in Earle's buffer pH 7.4 and filtered through two layers of cheesecloth (final dilution 1 : 300 w/v). The cerebral cortex and heart were prepared as above in Na^+/Mg^{2+} HEPES buffer pH 7.4 and diluted 1 : 150 w/v and 1 : 100 w/v, respectively. Previous experiments indicated that the binding behavior of the investigated compounds was the same in the two buffers used.

Binding curves for the different compounds were derived indirectly from competition experiments against a low concentration (0.5 nM) of ^{3}H-PZ to selectively label the M_1 receptor of the cerebral cortex and 0.3 nM ^{3}H-NMS for the other tissues. 1 ml of homogenate was incubated for 45 min at 30° in the presence of the labeled ligand and different concentrations of the cold ligand. Equilibrium was reached under these conditions as determined by appropriate experiments. The incubation was stopped by centrifugation (12,000 rpm for 3 min) at room temperature using an Eppendorf microcentrifuge. The resultant pellet was washed with 3 × 1.5 ml saline to remove the free radioactivity and the final pellet was allowed to drain. The tips of the tubes containing the pellets were cut off and 200 μl of tissue solubilizer (Lumasolve, Lumac) were added and left to stand overnight. Radioactivity was then counted after addition of 4 ml of liquid scintillation mixture (toluene : dimilume 10 : 1 v/v, Packard).

Assays were carried out in triplicate or quadruplicate and the non-specific binding was defined as the radioactivity bound or entrapped in the pellet when the incubation medium contained 1 μM QNB in [^{3}H]-NMS experiments and 1 μM ATR in [^{3}H]-PZ experiments. Non-specific binding averaged less than 10% and 30% of total radioactivity respectively.

Statistical analyses. The corrected IC_{50} values (K_Ds) were obtained by non-linear regression analysis on the basis of a one binding site model with a TOPFIT-pharmacokinetic program package [9] after correction for the radioligand occupancy shift according to the equation:

$$IC_{50} \text{ corrected} = IC_{50}/1 + {}^*C/{}^*K_D$$

where $^*C$ and $^*K_D$ represent the concentration and the dissociation constant of the radioligand used, respectively [10].

Hill coefficients (n_H) were calculated by linear regression analysis and statistically assessed.

For ^{14}C -AP uptake studies, data are expressed as the ratio of ^{14}C -AP accumulated in parietal cells to that in the medium. This accumulation ratio (AR) was determined by the following expression:

$$AR = (dpm_p/\text{parietal cell volume})/dpm_m$$

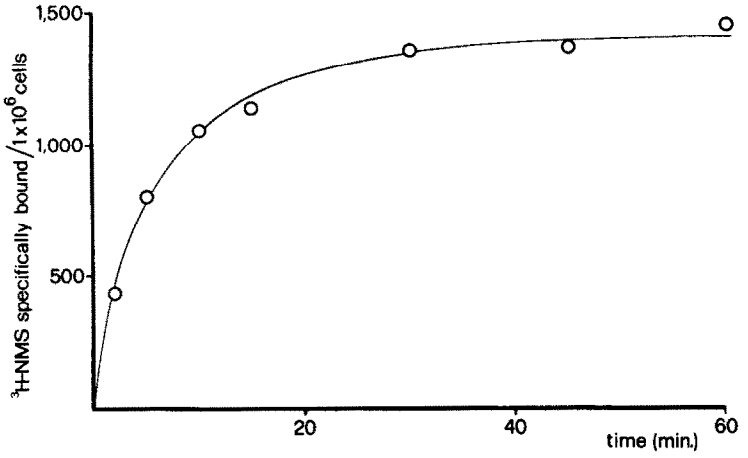

Fig. 1. Time course of association of [^{3}H]-NMS to muscarinic receptors in gastric fundic cells. Cells (5×10^6 cells/ml) were incubated with 0.4 nM [^{3}H]-NMS at 30°. Samples were filtered at the indicated times. Each point was determined in quadruplicate and represents the radioactivity specifically bound to 1×10^6 cells.

where dpm_p are the total disintegrations in the cell pellet, dpm_m are the disintegrations per milliliter of incubation medium; the parietal cell volume was calculated as described [11].

Data from the dose–response studies were normalized as the percentage of the maximal response. pA_2 values were calculated by Schild plot regression analysis [12].

RESULTS

Time course of ^{3}H-*NMS binding*

The specific binding of 0.4nM [^{3}H]-NMS to GFC was maximal after 30 min incubation at 30° without significant decrease up to 60 min (Fig. 1). Half-time of association was about 4 min. In subsequent binding experiments incubation was performed for 45 min at 30°.

Binding studies in gastric fundic cells

Occupancy–concentration curves for the different antagonists, were obtained indirectly from competition experiments against 0.4 nM[^{3}H]-NMS. The binding behavior did not significantly deviate from the simple Langmuir isotherm ($nH \simeq 1$) for any of the compounds tested as illustrated in Fig. 2. The rank order of potency was NMS > ATR ⋙ HHSiD > PZ with affinity values indicated in Table 1.

Binding studies in tissue homogenates

As indicated in Table 2 the binding behavior of the different compounds in the tissues studied was adequately described by the simple Langmuir isotherm ($nH \simeq 1$).

NMS displayed only slight variation (0.46–0.58 nM) in its affinity for the muscarinic receptors in the different tissues. ATR also showed relatively

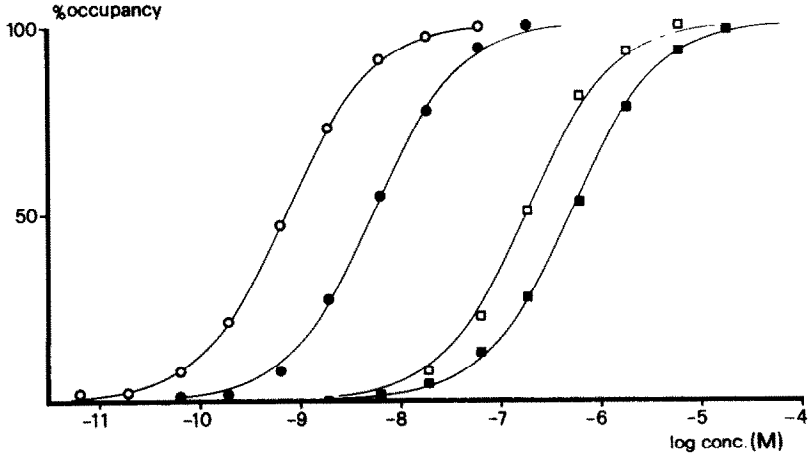

Fig. 2. Occupancy–concentration curves in gastric fundic cells. Binding curves for NMS (○), ATR (●), HHSiD (□) and PZ (■) were derived indirectly against 0.4 nM [^{3}H]-NMS and represent the mean of at least three experiments performed in quadruplicate.

Table 1. Antagonist affinity values for the gastric fundic cell muscarinic receptor derived from functional tests and binding experiments

Antagonist	*pA_2	†K_D(nM)	pK_D
NMS	10.30(10.60–10)	0.82 ± 0.175	9.09
ATR	9.20(9.45–8.95)	6.30 ± 0.953	8.20
HHSiD	7.10(7.35–6.85)	211 ± 13.58	6.68
PZ	6.25(6.43–6.08)	536 ± 26.42	6.27

*The data represent the mean of two experiments run in triplicate. The individual values are shown in brackets.

†The data represent the means ± SEM of three experiments run in quadruplicate.

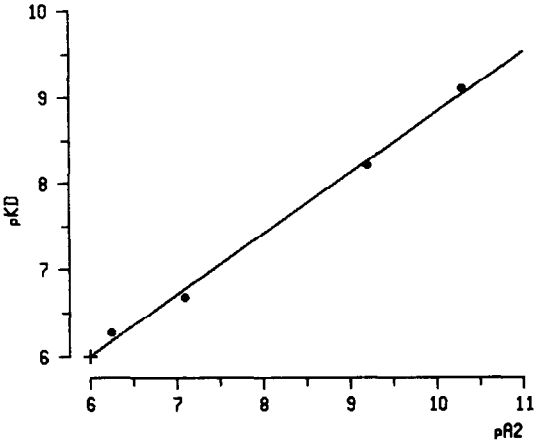

Fig. 3. Correlation between pA_2 and pK_D values in gastric fundic cells. Correlation was assessed by linear least squares regression analysis (r^2 = 0.98).

small variation, its affinity values ranging from 1.82 nM (cerebral cortex) to 11.6 nM (heart).

By contrast, PZ and HHSiD exhibited a selective behavior but the two drugs demonstrated different patterns in their selectivity profile. Whereas PZ showed a high affinity for the M_1 receptor of the cerebral cortex (13 nM) and low affinity for the M_2 receptors of the gland (424 nM) and heart (555 nM), HHSiD exhibited high affinity for both the M_1 subtype and the glandular M_2 receptor (K_Ds of 16 nM and 31 nM, respectively) and low affinity for the cardiac M_2 receptor (K_D of 331 nM). The rank order of potency in the submandibular gland (Fig. 4 and Table 2) was the same as in GFC.

^{14}C-aminopyrine accumulation

Carbachol stimulated [^{14}C]-AP accumulation in parietal cells in a concentration-dependent manner over the range of 0.1–10 μM. The addition of the tested compounds to the medium produced parallel rightward shifts of the carbachol concentration–response curve (data not shown). The calculated pA_2 values indicated that NMS and ATR were highest in potency (10.3 and 9.2, respectively), HHSiD was intermediate (7.10) and PZ exhibited the weakest potency (6.25) (Table 1). As shown in Fig. 3, the results correlated (r^2 = 0.98) with the affinity values found in the binding studies.

DISCUSSION

Pirenzepine is the first antimuscarinic compound known to selectively inhibit acid secretion in animals and man, producing only minor side-effects of the anticholinergic class of drugs. *In vivo* studies indicate that its potency for inhibition of acid secretion is within the same order of magnitude as atropine, a potent but non-selective antimuscarinic agent [13].

Occupancy–concentration curves for PZ to muscarinic receptors of GFC did not significantly deviate from the simple Langmuir isotherm (nH values close to 1) indicating that PZ binds to a homogeneous population of sites. These binding sites showed low affinity for PZ, the dissociation constant being 536 nM. Furthermore, PZ displays low affinity in the ^{14}C -AP test (pA_2 = 6.25) which is in very good agreement with the K_D value found in binding studies. This suggests that the receptor sites in the GFC are of the M_2 muscarinic receptor subtype which was confirmed by the absence of high affinity (M_1) binding sites as indicated by experiments performed with low concentration of ^{3}H -PZ.

PZ inhibits acid secretion induced by reflex nervous activity to a much greater extent than that caused by other stimuli, suggesting an antagonism at the neuronal level rather than at the effector cell level [14–18]. This datum is suggestive of an action mediated by the M_1 receptor [4]. The localization of these sites in the stomach is not known, but it has been proposed that they are present in the ganglia of the myenteric plexus of the stomach. Both the biochemical and functional results thus identify the homogeneous population of muscarinic receptors on the parietal cell as being of the M_2 subtype. The M_2 character of the receptor in GFC was verified by

Table 2. K_D values (nM) for the muscarinic receptor in submandibular gland, heart and cerebral cortex derived from binding experiments

Antagonist	Submandibular gland	Heart	Cerebral cortex (M_1)
NMS	0.46 ± 0.070(3)	0.54 ± 0.057(3)	0.58 ± 0.075(4)
ATR	3.70 ± 0.187(3)	11.6 ± 0.395(4)	1.82 ± 0.232(3)
HHSiD	30.6 ± 2.53(4)	331 ± 26.99(3)	15.7 ± 1.034(3)
PZ	424 ± 48.65(4)	555 ± 60.96(3)	13.2 ± 1.608(3)

The data represent the means ± SEM of (N) experiments run in triplicate.

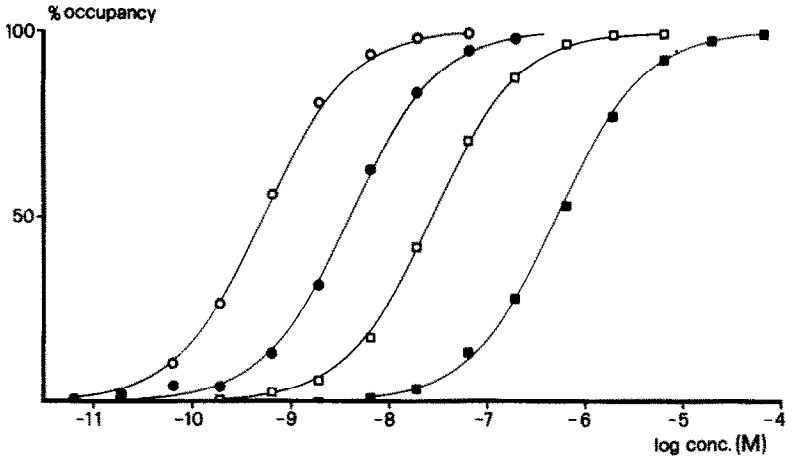

Fig. 4. Occupancy–concentration curves in submandibular gland homogenates. Binding curves for NMS (○), ATR (●), HHSiD (□) and PZ (■) were derived indirectly against 0.3 nM [^{3}H]-NMS and represent the mean of three experiments performed in triplicate.

further binding studies performed in submandibular gland, heart and cerebral cortex of the rabbit. In these tissues, PZ exhibited both the same affinity range, highest in cerebral cortex, intermediate in submandibular gland and lowest in heart, and selectivity ratio previously described for other species [2, 3, 19, 20].

On the basis of earlier results obtained by Lambrecht and Mutschler [6] in functional tests with HHSiD, it was suggested that the M_2 receptors are heterogeneous. The present data add further support for this hypothesis: HHSiD shows higher affinity for the glandular M_2 than for the cardiac M_2 muscarinic receptor subtype. However, differently from PZ, HHSiD was not able to discriminate between the cortical M_1 and the glandular M_2 receptor.

Whereas it appears that the M_1 represents a homogeneous class of receptors, evidence suggesting subclassification of the M_2 receptors is becoming compelling and has recently been substantiated by the finding that a new cardioselective competitive muscarinic antagonist (AF-DX 116) is able to discriminate between M_2 cardiac and M_2 glandular receptors of a factor of about 35 [21].

Acknowledgements—We wish to thank Dr G. B. Schiavi for his helpful contributions, Mr J. Toffetti and Ms L. Monti for their technical assistance, Ms C. Pelanda for performing the statistical analyses and Ms E. Borghi for typing the manuscript (Istituto De Angeli).

This project was supported by grants from Boehringer Ingelheim (France and Italy) and from Le Centre de la Recherche Scientifique (France).

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