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M_3-SUBTYPE MUSCARINIC RECEPTOR THAT CONTROLS INTRACELLULAR CALCIUM RELEASE AND INOSITOL PHOSPHATE ACCUMULATION IN GASTRIC PARIETAL CELLS

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Abstract—The muscarinic receptor subtype which triggers acid secretion was investigated in isolated rabbit gastric parietal cells. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), measured with the fluorescent indicator FURA-2, increased rapidly after full agonist (carbachol) stimulation (6–8 sec), then returned to an intermediate sustained value. Other M_2-agonists, oxotremorine and arecoline, produced a partial $[Ca^{2+}]_i$ increase, whereas M_1-agonists, pilocarpine and [4-*m*-chlorophenylcarbamoyloxyl]-2-butynyl-trimethylammonium, were without any significant effect. $[Ca^{2+}]_i$ rise was inhibited by selective muscarinic antagonists: atropine > 4-diphenylacetoxy-*N*-methyl-piperidine methbromide > quinuclidinylbenzilate (QNB) > pirenzepine > 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one, this sequence being characteristic of the involvement of an M_3-subtype. This inhibition was shown to be stereoselective; dexetimide and (−)QNB were more potent than levetimide and (+)QNB. The IC_{50} values for inhibition of $[Ca^{2+}]_i$ increase by muscarinic antagonists were in good agreement with those obtained for inhibition of phospholipase C activation. In conclusion, the muscarinic receptor that controls acid secretion appears to be of the M_3-subtype and the biochemical events coupled to the activation of this receptor system are also controlled through the same subtype.

Muscarinic receptor activation involves several effector mechanisms: activation of the phosphoinositide turnover, inhibition of adenylate cyclase, activation of guanylate cyclase, activation of K^+/Ca^{2+}-dependent channels, inhibition of M-currents and activation of Ca^{2+} channels [1]. Binding studies with new specific muscarinic antagonists provided evidence for the existence of a heterogeneous muscarinic receptor system. A first classification of two different subtypes was proposed; they were defined by their respective affinity for the antagonist pirenzepine: M_1 with affinity in a nanomolar range and M_2 with lower affinity in a micromolar range [2]. A further classification was proposed from binding experiments with 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*] [1,4]benzodiazepine-6-one (AFDX-116) and methoctramine which showed high affinity for the M_2-subtype (cardioselective), in contrast to 4-diphenylacetoxy-*N*-methyl-piperidine methbromide (4-DAMP) and hexahydrosiladifenidol (HHSiD) which showed high affinity for the M_3-subtype (exocrine glands and smooth muscles) [3–6]. Molecular biological studies confirmed the presence of various subtypes: five different proteins were described as the products of five distinct genes. The effector systems coupled to these subtypes have been partially described [7, 8].

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Muscarinic agents stimulate *in vitro* aminopyrine accumulation in gastric parietal cells or glands [9, 10]. Binding studies led us to define, in rabbit parietal cells, the presence of an M_2-subtype coupled to aminopyrine accumulation [11]. In rabbit [12] or rat [13] parietal cells, activation of the muscarinic receptor by carbachol (CCh) was shown to be coupled to polyphosphoinositide hydrolysis. More recently, Pfeiffer *et al.* [14], in rat gastric parietal cells, demonstrated the presence of an M_3-muscarinic receptor subtype coupled to phosphoinositide hydrolysis and aminopyrine accumulation. Chew and Brown [15], Negulescu and Machen [16] in the rabbit and Muallem and Sachs [17] in the dog, demonstrated that CCh activation of the receptor causes changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$).

In this paper, using isolated rabbit gastric parietal cells, we investigated phosphoinositide hydrolysis and intracellular Ca^{2+} release following activation of the muscarinic receptor subtype involved in gastric acid secretion.

MATERIALS AND METHODS

Sources of materials. [4-*m*-Chlorophenylcarbamoyloxyl]-2-butynyl-trimethylammonium (McN-A-343), quinuclidinylbenzilate (QNB) and AFDX-116 were from Laboratoires Boehringer-Ingelheim (France), 4-DAMP, carbachol, atropine, acetylcholine (ACh), oxotremorine, pilocarpine, arecoline, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethane-

sulfonic acid (HEPES), ethyleneglycol-bis(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), FURA-2 AM and bovine serum albumin (fraction V) (BSA) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{14}C]-Aminopyrine (80 Ci/mmol) and *myo*-[2-^{3}H]inositol (10–20 Ci/mmol) were from Amersham (U.K.). Collagenase (0.8 units/mg, from *Clostridium histolyticum*) was from Serva (Heidelberg, F.R.G.). Earle's balanced salt solution was from Biomérieux (France). Medium A consisted of Earle's balanced salt solution without bicarbonate containing 10 mM HEPES and 0.2% BSA, pH 7.4.

Preparation of isolated rabbit gastric parietal cells. Cell isolation was carried out following the collagenase/EDTA procedure as described previously [18]. Cell separation was performed by counterflow centrifugation with a Beckman elutriator rotor JE6-B [19]. Three fractions were collected at a rotor speed of 2100 rpm by increasing the flow rate from 24 to 44 and 68 mL/min. This third fraction contained mainly parietal cells (70%), some clumps of mucus cells and a few chief cells (15%) which could be responsible, at least in part, for an overestimation of both $[Ca^{2+}]_i$ and inositol phosphate measurement but not of aminopyrine (AP) accumulation measurement. Cell viability (trypan blue exclusion) was always greater than 95%.

Measurement of inositol phosphate accumulation. Cells (2×10^7 per mL) were incubated for 2.5 hr at 37° in oxygenated (95% O_2/5% CO_2) medium A with 40 μCi/mL [^{3}H]*myo*-inositol, as described previously [12]. After two washings with the same medium, cells were equilibrated for 20 min at 37° in the presence of 10 mM LiCl. Immediately after washing, cells (5×10^6 per sample) were incubated for 20 min at 37° with continuous stirring and gassing (95% O_2/5% CO_2) in the absence or presence of agents. The reaction was stopped by a rapid addition of 1 mL of 10% $HClO_4$. Inositol phosphate was analysed on a Dowex anion exchange column.

Cytosolic free Ca^{2+} *concentration measurement.* The fluorescent probe FURA-2 AM was used to assess $[Ca^{2+}]_i$ as described by Chew and Brown [15]. Cells (2.5×10^6 per mL) were incubated for 20 min at 37° with 2 μM FURA-2 AM in medium A. Cells

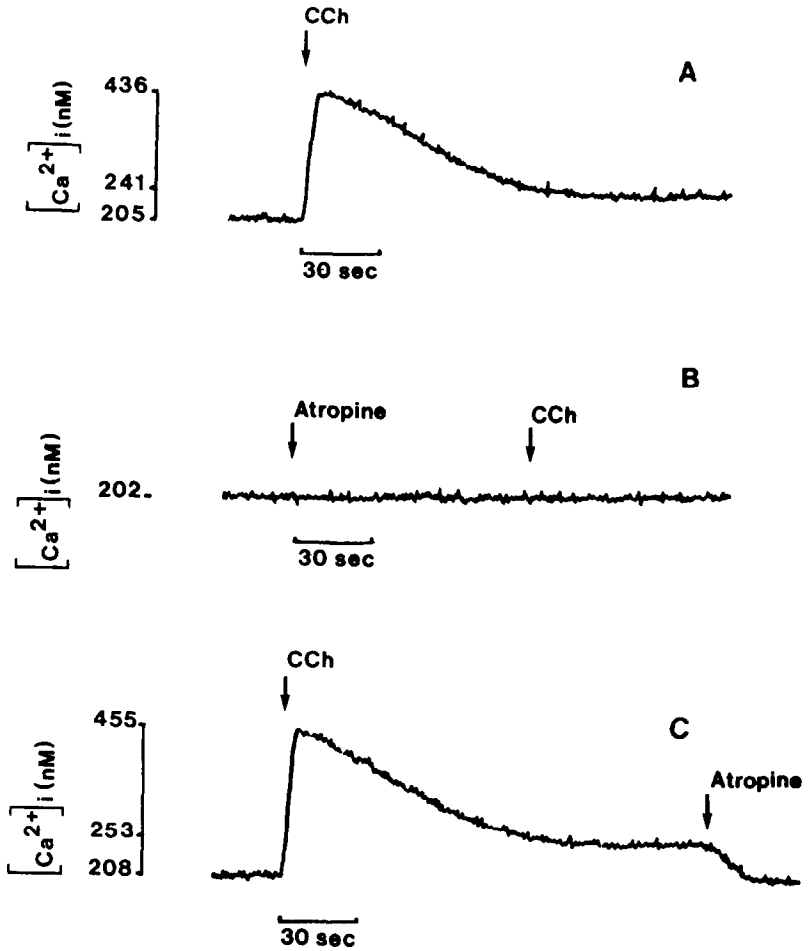

Fig. 1. Time-course of effects of CCh on $[Ca^{2+}]_i$. Cells (2.5×10^6 per mL) were incubated for 20 min at 37° with 2 μM FURA-2 AM. After washing, cell fluorescence (340 nm excitation, 510 nm emission) was recorded as a function of time and 0.1 mM CCh was added. $[Ca^{2+}]_i$ was expressed in nM as indicated in Materials and Methods. Atropine (100 nM) was added (B) when the plateau value following CCh stimulation was reached or (C) 1 min before addition of CCh.

were then washed by centrifugation (2 min at 100 *g*) with BSA-free medium A and cell pellets were resuspended in 3 mL BSA-free medium A. Agents were added at the time of the fluorescence measurement. Fluorescence measurements were performed using a KONTRON SFM 25 spectrofluorimeter. The excitation and emission wavelengths were 340 and 510 nm, respectively. Subsequently the cells were lysed by adding Triton X-100 (final concentration 0.3%) to obtain the signal of the Ca^{2+}-saturated dye (F_{max}). F_{min} for the Ca^{2+}-free form of the dye was recorded by adding 10 mM EGTA at pH 8.5. $[Ca^{2+}]_i = K_d\ (F - F_{min}/F_{max} - F)$ where $K_d = 224$ nM according to Grynkiewicz *et al.* [20].

Curve fittings and statistical evaluations. Data from the dose–response studies were normalized as percentage of maximal response. Values are means ± SEM from separate experiments (see figure legends). Hill coefficients (n_H) were computed from linear regression analysis and assessed statistically.

RESULTS

Changes in $[Ca^{2+}]_i$ *following muscarinic receptor activation*

Figure 1A reports the variations in $[Ca^{2+}]_i$ following activation of isolated parietal cells by CCh (1 mM). The elutriated cell fraction used in these experiments contained about 70% parietal cells. CCh caused a rapid increase in $[Ca^{2+}]_i$ from 207 ± 8 nM (N = 10) (basal value) up to 442 ± 14 nM (maximum within 6 sec). After this transient response, $[Ca^{2+}]_i$ returned to an intermediate plateau value of 240 ± 15 nM within 1.5 min. The transient response was due to the release of Ca^{2+} from internal stores, whereas the plateau value was due to a Ca^{2+} influx through plasma membrane as only the latter was abolished in a Ca^{2+}-depressed medium (data from Ref. 21).

When the competitive muscarinic antagonist atropine (0.1 μM) was added before CCh, no variation in $[Ca^{2+}]_i$ was observed (Fig. 1B). If added during the sustained response to CCh, $[Ca^{2+}]_i$ returned to basal value within 5 to 10 sec (Fig. 1C).

Effect of muscarinic agonists on $[Ca^{2+}]_i$

The muscarinic agonists, ACh and CCh, induced a transient dose-dependent $[Ca^{2+}]_i$ increase in isolated rabbit parietal cells. ACh and CCh stimulated $[Ca^{2+}]_i$ increase with the same efficacy but the ACh K_{act} value was 10 times lower than the CCh K_{act} value: 0.25 ± 0.04 and 3.2 ± 0.7 μM, respectively (Fig. 2). The other agonists, arecoline (0.1 mM) and oxotremorine (0.1 mM), caused only a partial response (44 ± 5% increase of the response

Table 1. Effects of the various muscarinic agonists on the $[Ca^{2+}]_i$ response

Muscarinic agonist	% Maximal response to 0.1 mM CCh
Acetylcholine	96 ± 2
Oxotremorine	44 ± 5
Arecoline	43 ± 2
Pilocarpine	3 ± 2
McN-A-343	0

Cells (2.5×10^6 per mL) were incubated for 20 min at 37° with 2 μM FURA-2 AM. After washing, cell fluorescence (340 nm excitation, 510 nm emission) was recorded as a function of time in the presence of 0.1 mM agonists (N = 4) and expressed as a percentage of the maximal response to 0.1 mM CCh.

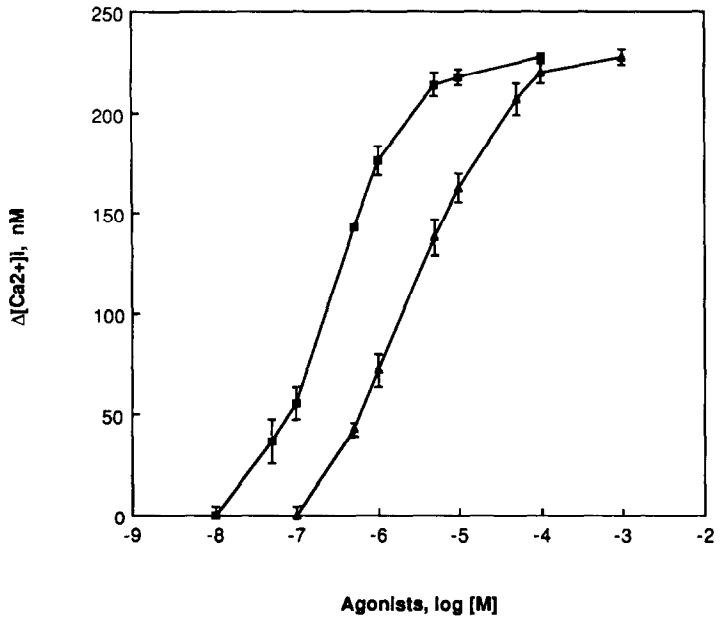

Fig. 2. Effects of ACh and of CCh on $[Ca^{2+}]_i$ levels. $[Ca^{2+}]_i$ measurements were assessed as indicated in Fig. 1. Results, mean ± SEM of 6 separate experiments for ACh (■) and 4 experiments for CCh (△), were expressed as nM $[Ca^{2+}]_i$.

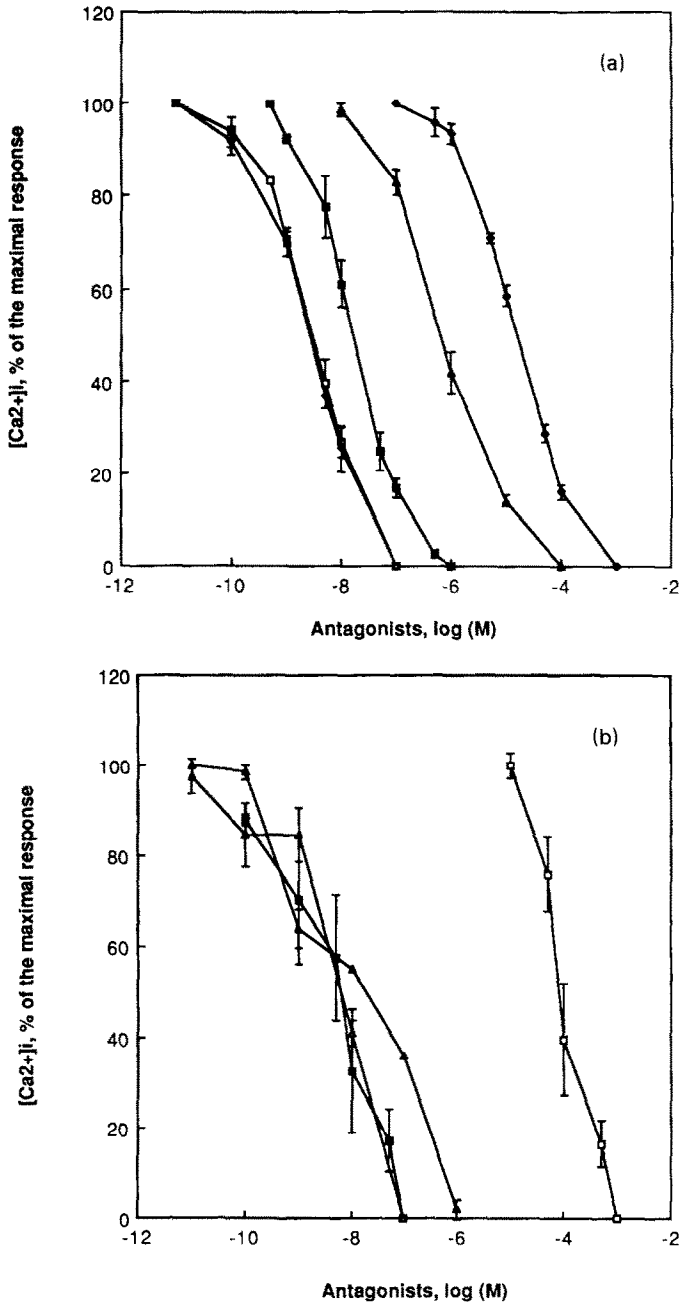

Fig. 3. Effects of muscarinic antagonists on CCh-induced $[Ca^{2+}]_i$ levels. $[Ca^{2+}]_i$ measurements were assessed as indicated in Fig. 1. Antagonists were added to the medium 1.5 min before addition of 0.1 mM CCh. Results, mean ± SEM from 4 separate experiments, are expressed as percentage of the maximal response to CCh. (a) Atropine (◇), QNB (■), 4-DAMP (□), pirenzepine (▲), AFDX-116 (ǁ); (b) dexetimide (■), levetimide (□), QNB (+) (△), QNB (−) (▲).

to 1 mM CCh for arecoline and 43 ± 2% for oxotremorine). In contrast, the $[Ca^{2+}]_i$ increase induced by 0.1 mM pilocarpine was very low (3 ± 2%) and 0.1 mM McN-A-343 had no effect (Table 1).

Effect of muscarinic antagonists on $[Ca^{2+}]_i$

All muscarinic antagonists inhibited dose-dependently CCh-induced $[Ca^{2+}]_i$ rise in isolated rabbit parietal cells (Fig. 3). 4-DAMP (M_3-antagonist), atropine and QNB (non-selective antagonists) were the most potent inhibitors, as indicated in Table 2. Compared with these agents, pirenzepine (M_1-antagonist) and AFDX-116 (M_2-antagonist) were less potent: IC_{50} values were 250 times (pirenzepine) and 5800 times (AFDX-116) lower than that of atropine (Table 2 and Fig. 3). Hill coefficients did not differ significantly from $n_H = 1.00$, in agreement with a single langmuir isotherm.

Table 2. IC_{50} values of inhibition of CCh-induced inositol phosphate accumulation and CCh-induced $[Ca^{2+}]_i$ increase by muscarinic antagonists

	$[Ca^{2+}]_i$ increase	Inositol phosphate accumulation
Atropine	2.6 ± 0.6	3.6 ± 2
4-DAMP	3.2 ± 0.8	5.4 ± 1.8
QNB (+)	20 ± 4	ND
QNB (−)	8 ± 4	4 ± 2
QNB	16.0 ± 4.0	7.3 ± 1.4
Pirenzepine	660 ± 18	440 ± 17
AFDX-116	15,000 ± 3000	8300 ± 170
Dexetimide	8 ± 2	ND
Levetimide	100,000 ± 4000	ND

IC_{50} values (nM) were determined from inhibition curves of CCh-induced (0.1 mM) inositol phosphate accumulation and from inhibition curves of CCh-induced (0.1 mM) $[Ca^{2+}]_i$ levels.
ND, not determined.

Correlation between muscarinic-dependent $[Ca^{2+}]_i$ *and total inositol phosphate*

CCh induced a dose-dependent rise in total inositol phosphate accumulation in isolated parietal cells (Fig. 4) with an EC_{50} value of 6.0 ± 1.7 μM. The basal value was 650 ± 180 dpm per million cells; 1 mM CCh caused a 2.5-fold increase in total inositol phosphate level (1720 ± 650 dpm per million cells) (N = 3). This CCh-induced response was inhibited dose-dependently by muscarinic antagonists in the range of affinities listed in Table 2. Hill coefficients did not differ significantly from $n_H = 1.00$, in agreement with a single langmuir isotherm. Figure 5 shows a close correlation ($r = 0.99$) between IC_{50} values for inhibition by muscarinic antagonists of CCh-induced transient increase in $[Ca^{2+}]_i$ and of CCh-induced total inositol phosphate accumulation.

DISCUSSION

We found previously that muscarinic receptor occupation was correlated with aminopyrine accumulation in the rabbit parietal cell and we suggested that this biological event resulted from an activation of the M_2-subtype [11]. We also showed that muscarinic receptor activation was followed by an increase in inositol phosphate [12], but no characterization of the subtype involved was given. The results concerning inositol phosphate accumulation were in agreement with those from Pfeiffer *et al.* [13] who demonstrated further the involvement of an M_2-subtype. In this paper, we attempted to define clearly, with the use of selective agonists and antagonists, the muscarinic receptor subtype that mediates acid secretion from isolated gastric parietal cells of the rabbit.

Intracellular $[Ca^{2+}]$ measurements with the fluorescent indicator FURA-2 showed that CCh caused a rapid increase in $[Ca^{2+}]_i$ followed by a sustained response. The transient increase may have been due to the release of Ca^{2+} from intracellular calcium stores and the sustained response to a calcium influx through plasma membrane. These fluxes are directly dependent upon muscarinic receptor activation, as they were totally blocked by atropine. These results are in agreement with

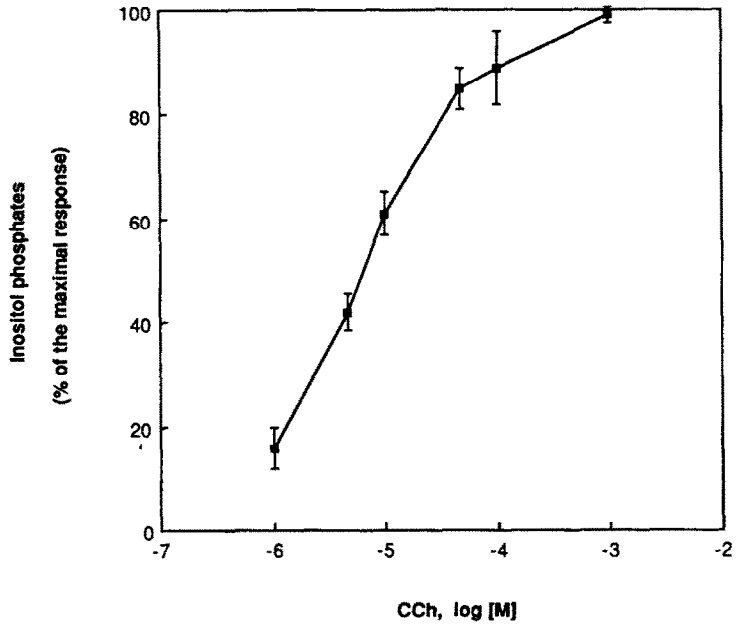

Fig. 4. Effects of CCh on total inositol phosphate accumulation. Total inositol phosphate was determined in parietal cells incubated for 2.5 hr at 37° with 40 μCi/mL *myo*-[^{3}H]inositol and, after washing, for an additional 20 min with 10 mM LiCl. Various concentrations of CCh were added for an additional 20 min in the presence of 10 mM LiCl. Radiolabeled inositol phosphate was separated by ion-exchange chromatography on DOWEX 1X8. Results, mean ± SEM from 3 separate experiments run in triplicate, are expressed as percentages of the maximal response to 1 mM CCh.

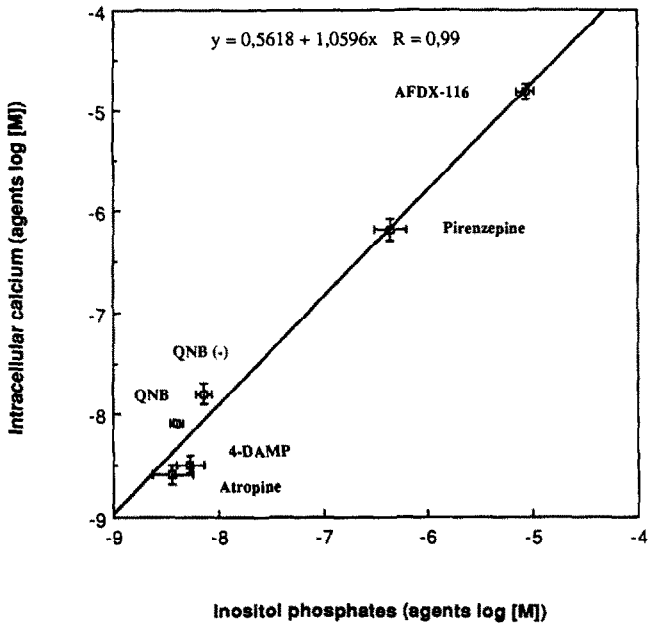

Fig. 5. Correlation between inhibition of CCh-induced inositol phosphate accumulation and CCh-induced $[Ca^{2+}]_i$ increase by muscarinic antagonists. IC_{50} values (means ± SEM) were determined from inhibition curves by muscarinic antagonists (atropine, QNB (−), QNB racemic, 4-DAMP, pirenzepine and AFDX-116) of CCh-induced (0.1 mM) inositol phosphate accumulation and of CCh-induced (0.1 mM) $[Ca^{2+}]_i$ levels. Parameters of the mean square regression line were computed from individual values and reported on the graph.

previous data [15, 16]. Partial agonists, oxotremorine and arecoline, which stimulate M_2-subtype muscarinic receptors in several tissues [22], caused a partial increase in $[Ca^{2+}]_i$, whereas pilocarpine and McN-A-343, agonists for M_1-subtype [22, 23], were without any significant effect. This suggests the involvement of an M_2-subtype muscarinic receptor which controls Ca^{2+} fluxes. The use of a selective antagonist, pirenzepine, led us to confirm this hypothesis: in contrast to brain, where pirenzepine interacts with high affinity with an M_1-muscarinic receptor, the inhibition of CCh-induced $[Ca^{2+}]_i$ increase was obtained with elevated concentrations of pirenzepine (0.1 μM), in agreement with the presence of an M_2-subtype. In contrast, the non-selective antagonist, atropine, inhibited this increase at low concentrations (1 nM). HHSiD was found to be selective for the M_3-subtype. However, previous results from Baudière *et al.* [24] using the same model have shown that the affinity of HHSiD for gastric muscarinic receptor (K_D = 211 nM) was lower than its affinity for submandibular gland muscarinic receptor ($K_D \approx$ 30 nM). This discrepancy led us to choose the other M_3-selective antagonist, 4-DAMP, which we found to inhibit $[Ca^{2+}]_i$ increase in a nanomolar range, whereas AFDX-116, selective for the M_2-subtype, inhibited this increase in a micromolar range. So, $[Ca^{2+}]_i$ elevation, due to the activation of a muscarinic receptor in parietal cells, was inhibited in the following order of potency: atropine > 4-DAMP > QNB > pirenzepine > AFDX-116.

This sequence was shown to be characteristic of an M_3-subtype found in exocrine glands and smooth muscles. Moreover, dexetimide and (−)QNB were more potent than levetimide and (+)QNB, in agreement with a stereoselective interaction of these antagonists with the M_3-muscarinic receptor subtype.

Similar results have been obtained in parotid glands and in pancreatic acini where the receptor involved in the activation of the phospholipase C was of M_3-subtype [25, 26]. These data are in agreement with the results from molecular biological studies which showed the existence of a specific subtype in exocrine glands coupled to the phosphoinositide turnover and $[Ca^{2+}]_i$ increase [8, 27].

When comparing receptor occupation by muscarinic agonists and aminopyrine accumulation in parietal cells, it appears that the concentration required to get 50% occupation was higher than that needed to get a 50% increase in inositol phosphate formation, $[Ca^{2+}]_i$ release and AP accumulation. This finding suggests the presence of spare receptors in this cellular system (Table 3).

Table 3. Comparative effects of carbachol on isolated parietal cells

[^{3}H]NMS binding*	50.0 ± 5.0
Inositol phosphate accumulation	6.0 ± 1.7
$[Ca^{2+}]_i$ increase	3.2 ± 0.7
Aminopyrine accumulation*	5.2 ± 2.2

Carbachol concentrations (mean ± SEM, μM) that induced 50% inhibition of [^{3}H]NMS binding and stimulation of inositol phosphate accumulation, $[Ca^{2+}]_i$ increase and aminopyrine accumulation are reported on the table.

* From previous work by Magous *et al.* [11].

Moreover, a close relationship was found between the IC_{50} values for inhibition by muscarinic antagonists of CCh-induced phospholipase C activity and CCh-induced $[Ca^{2+}]_i$ increase.

In conclusion, we demonstrated in this paper that the muscarinic receptor which controls acid secretion from gastric parietal cells was of M_3-subtype (exocrine gland and smooth muscle subtype) and we showed that the biochemical events (activation of the phospholipase C and $[Ca^{2+}]_i$ increase) were coupled to the same receptor subtype.

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