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Characterization of pre- and postsynaptic muscarinic receptors in circular muscle of pig gastric fundus

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1 This study investigated the subtype of muscarinic receptors on the cholinergic neurones and smooth muscle in the circular muscle of the pig gastric fundus.

2 Muscarinic antagonists, except MT-3, concentration-dependently inhibited the contractions induced by a given concentration of acetylcholine. Concentration-response curves by acetylcholine were shifted rightwards in a parallel manner without depression of the maximum by the muscarinic antagonists, except by MT-3 that induced a leftward shift. Correlation of the pIC_{50} and pA_2 values with published pK_i values for the five muscarinic receptor subtypes suggests that the muscarinic receptors on pig gastric fundus circular muscle belong to the M_3 subtype.

3 Electrically-evoked contractions (40 V, 4 Hz, 0.25 ms, 2 min) were concentration-dependently inhibited by the muscarinic antagonists except for methoctramine and AF-DX 116, that increased the amplitude of the electrically-induced contractions in lower concentrations. MT-3 tended to increase the electrically-induced contractions.

4 The antagonists, except MT-3, concentration-dependently increased the electrically-induced tritium outflow (40 V, 4 Hz, 0.25 ms, 2 min) after incubation of the tissues with [^{3}H]-choline. MT-3 (3×10^{-8} and 10^{-7} M) decreased the electrically-induced tritium release. Correlation of the pIC_{50} values with published pK_i values for the different muscarinic receptor subtypes yielded a significant and comparable correlation for M_1, M_3, M_4 and M_5 receptors.

5 These results suggest that the postsynaptic receptors in circular muscle of the pig gastric fundus belong to the M_3 subtype. However, the presynaptic receptor could not be clearly defined, although it does certainly not belong to the M_2 subtype.

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Keywords: Pig gastric fundus; muscarinic receptor; smooth muscle; presynaptic receptors

Abbreviations: 4-DAMP, 4-diphenyl-acetoxy-N-piperidine; AF-DX 116, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido [2,3b][1,4]-benzodiazepine-6-one; EFS, electrical field stimulation; LMMP, longitudinal muscle myenteric plexus; L-NAME, N^G-nitro-L-arginine methyl ester; MT-3, mamba toxin-3; p-F-HHSiD, *para*-fluorohexahydrosiladiphenidol; PSS, physiological salt solution; TTX, tetrodotoxin

Introduction

In circular muscle strips of the pig gastric fundus, contractions induced by electrical field stimulation (EFS) are blocked by atropine (Leclere & Lefebvre, 1998), while the electrically-evoked release of acetylcholine in the same tissue is enhanced by atropine (Leclere & Lefebvre, 2001), indicating the presence of respectively postsynaptic muscular muscarinic receptors and presynaptic inhibitory muscarinic auto-receptors on the cholinergic neurones of the pig gastric fundus. However, the type of muscarinic receptor(s) involved in the pig gastric fundus has not yet been characterized. Five different muscarinic receptor subtypes have been identified based on studies of molecular structure, *in vitro* binding and function (Buckley *et al*., 1989; Dörje *et al*., 1991). All subtypes belong to the seven-transmembrane G-protein coupled receptor family (see reviews Grimm *et al*., 1994; Eglen *et al*., 1996; Caulfield & Birdsall, 1998). In most tissues, including the gastrointestinal tract, mainly M_3 receptors can be detected to function pharmacologically at the postsynaptic level (Eglen *et al*., 1996; Caulfield & Birdsall, 1998). With regard to the presynaptic level, the situation is more complex as both stimulatory and inhibitory muscarinic auto-receptors can be present, and only a limited number of experiments have been reported in the gastrointestinal tract. In guinea-pig longitudinal muscle myenteric plexus (LMMP) preparations, it has been shown that presynaptic muscarinic M_3 receptors inhibit while muscarinic M_1 receptors enhance acetylcholine release (Soejima *et al*., 1993). However, in circular smooth muscle of the guinea-pig ileum, the presynaptic inhibitory muscarinic receptors belong to the M_1 subtype (Dietrich & Kilbinger, 1995). In the guinea-pig stomach, there is evidence that acetylcholine release is inhibited by muscarinic M_1 and M_2 receptors (Ogishima *et al*., 2000). In canine LMMP preparations, a binding study demonstrated that presynaptic muscarinic receptors belong to the M_3 subtype, although this technique could not exclude the presence of another presynaptic subtype (Kostka *et al*., 1989). Prejunctional stimulatory and inhibitory muscarinic receptors have also been demonstrated in other tissues, especially in the respiratory tract and urinary bladder (see reviews Grimm *et al*., 1994; Somogyi & de Groat, 1999).

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By use of functional and release experiments, the present study had two objectives: First, to characterize the muscular muscarinic receptor, responsible for the contraction by muscarinic agonists of the smooth muscle cells in the pig gastric fundus. Second, to characterize the muscarinic receptor(s) present on the cholinergic nerves, inhibiting the release of acetylcholine.

Methods

Tissue preparation

Experiments were carried out on isolated circular smooth muscle strips of the pig gastric fundus. The stomach was removed from healthy castrated male pigs, slaughtered at a local abattoir, and transported to the laboratory in ice-chilled physiological salt solution (PSS). After the mucosa was removed, strips of approximately 1.5 cm in length and 0.3 cm in width were cut in the direction of the circular muscle, except in one series of experiments when strips were cut in the direction of the longitudinal muscle. In one series, circular muscle strips were prepared after removal of the longitudinal muscle layer and the myenteric plexus. All strips were used within 24 h. When tissues were used the next day, they were stored in fresh PSS at 4°C. Strips were mounted vertically between two platinum plate electrodes ($30 \times 6 \times 0.1$ mm) in 20 ml organ baths (functional experiments) or between two platinum wire electrodes (45×0.5 mm) in 2 ml organ baths (functional or release experiments) under a load of 2 g, containing PSS (mM): NaCl 112, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.5 and $NaHCO_3$ 25, maintained at 37°C and gassed with carbogen. The PSS contained guanethidine (4×10^{-6} M) to avoid noradrenergic influences, N^G-nitro-L-arginine methyl ester (L-NAME, 3×10^{-4} M) to prevent relaxations due to nitric oxide, and, as the basal tonus increases during the course of the experiment (Leclere & Lefebvre, 1998), indomethacin (10^{-5} M) to prevent this increase. The mechanical activity of the preparations was recorded *via* isotonic transducers (T_3, Palmer Bioscience, U.S.A.) on a recorder (FWR 3701 Graphtec Linearcorder or MC6625 Graphtec Multicorder, Japan or Ankersmit, The Netherlands). Electrical field stimulation (EFS) was applied by means of a S88 stimulator (Grass, U.S.A.).

Functional experiments

The tissues were allowed to equilibrate for 90 min with rinsing every 15 min before starting the experiment. After the equilibration period, strips were first maximally contracted with 80 mM KCl, followed by rinsing every 10 min during 30 min.

In a first set of experiments, muscarinic antagonists were tested *versus* electrically and acetylcholine-induced contractions. After equilibration and KCl treatment, EFS (40 V, 0.25 ms, 4 Hz during 2 min) was applied, contracting the tissues. Three to five consecutive electrical stimulations at 10 min intervals were required before contractions remained stable. When the response of muscarinic antagonists *versus* exogenous acetylcholine was studied, a cumulative concentration-response curve for acetylcholine was performed after the electrical stimulations, to determine the concentration of acetylcholine inducing a contraction of similar amplitude as that induced by EFS (3×10^{-7} to 10^{-5} M). Tissues were then re-exposed to this concentration of acetylcholine. After contraction to EFS or exogenous acetylcholine, tissues were washed to re-establish the basal tone level. To study the influence of tetrodotoxin (TTX; 3×10^{-6} M), hexamethonium (5×10^{-4} M) and atropine (10^{-6} M) on the electrically-evoked contractions and on the contractions by exogenous acetylcholine, they were added for 30 min before tissues were stimulated again with either EFS or exogenous acetylcholine. To study the influence of muscarinic antagonists, EFS or addition of acetylcholine was repeated at 45 min intervals and increasing concentrations of muscarinic antagonists or solvent were added 30 min before EFS or acetylcholine addition. The response before the first addition of antagonist was used as control response (S_1).

In a second set of experiments, muscarinic antagonists were tested *versus* cumulative concentration-response curves of acetylcholine in order to determine pA_2 values for the postsynaptic muscarinic receptors. After the equilibration period and KCl treatment, tissues were exposed to 10^{-6} M of acetylcholine to test their viability and responsiveness. After washing until basal tone level was re-established, cumulative concentration-response curves to acetylcholine (3×10^{-8} to 10^{-2} M) were constructed using half-logarithmic dosing increments of acetylcholine. After construction of the first concentration-response curve, the preparation was washed for 45 min, until the tension returned to baseline. A muscarinic antagonist was then incubated for 30 min before the second concentration-response curve was obtained. This cycle was repeated four times with increasing concentrations of antagonist. Parallel control experiments without antagonist were performed under identical conditions. In another series of experiments, cumulative concentration-response curves to acetylcholine (3×10^{-8} to 10^{-3} M) or KCl (5×10^{-3} to 8×10^{-2} M) were constructed. After construction of the first concentration-response curve, the preparation was washed for 45 min, until the tension returned to baseline. The M_4-selective antagonist MT-3 was then incubated for 30 min before the second concentration-response curve to acetylcholine or KCl was obtained. Parallel control experiments without antagonist were performed under identical conditions. The control experiments allowed for any correction to be made for changes in sensitivity to acetylcholine or KCl.

Release experiments

Strips, weighing 148 ± 5 mg ($n = 82$), were mounted vertically between two platinum wire electrodes in 2 ml organ baths containing PSS. The PSS contained in addition to the composition mentioned under Tissue preparation 1.5×10^{-6} M choline and 5.7×10^{-5} M ascorbic acid. Baths were maintained at 37°C and gassed with carbogen. The tissues were superfused at a rate of 2 ml min^{-1} using a peristaltic pump (Gilson Minipuls, France) during 60 min. The strips were subjected to continuous EFS (40 V, 1 ms, 0.5 Hz) during the last 20 min. After the equilibration period, superfusion was stopped and the preparations were incubated for 30 min with [^{3}H]-choline (5 μCi ml^{-1}) during which the tissues were stimulated electrically (40 V, 1 ms, 2 Hz) in order to label their cholinergic transmitter stores.

After the labelling procedure, the strips were superfused (2 ml min^{-1}) for 90 min with PSS to remove loosely bound radioactivity. From now on the PSS contained in addition 10^{-5} M hemicholinium-3 to prevent the re-uptake of choline. After the washout period, strips were no longer superfused but the content of the organ bath, filled with 1 ml, was collected and replaced every 3 min. A total of 75 samples was collected. 0.5 ml of the samples was mixed with 2 ml of the scintillator containing solution Ultima Gold (Canberra Packard, U.S.A.). The strips were stimulated five times for 2 min, except in one series of experiments with MT-3 when strips were only stimulated four times (S_1-S_5; 40 V, 0.25 ms, 4 Hz), at 10 min (S_1, 4th sample), 58 min (S_2, 20th sample), 106 min (S_3, 36th sample), 154 min (S_4, 52nd sample), and 202 min (S_5, 68th sample) after the end of the washout period. Muscarinic antagonists were added in increasing concentrations 30 min before S_2-S_5, and they remained present until the next concentration was added, or until the end of the experiment. At the end of the experiment, tissues were blotted and weighed.

Radioactivity of all samples was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR, Canberra Packard, U.S.A.), and external standardization was used to correct for counting efficiency. Electrical stimulation induced an increase in tritium overflow, not only during stimulation, but also in three samples following that with stimulation. The stimulation-induced increase in tritium overflow was calculated from the difference between the total tritium release during stimulation plus the following three samples, and the calculated basal tritium overflow. Basal tritium overflow during the period of enhanced tritium overflow was calculated by fitting a regression line through the values of the three samples just before stimulation and the values of the three samples starting from where basal release was re-established.

Data analysis

Experimental data are expressed as means ± s.e.mean and n refers to the number of the tissues from different animals. For both functional and release experiments, concentration-response curves for the muscarinic antagonists were constructed by expressing the ratio S_n/S_1 in the presence of an antagonist as a percentage of the equivalent ratio obtained in parallel tissues in the absence of antagonists. The concentrations which produced half-maximal inhibition of contractions or facilitation of tritium release (IC_{50}) were calculated by linear interpolation from individual concentration-response curves. Statistical significance ($P<0.05$) was assessed by the paired and unpaired t-test.

In experiments to determine the pA_2 values, the 2nd to 5th concentration-response curves to acetylcholine were expressed as percentage of the maximal contraction in the first concentration-response curve. The EC_{50} was calculated for each curve and the dose ratios (DR) were calculated as $(EC_{50})_n/(EC_{50})_1$. As a moderate rightward shift of the concentration-response curves to acetylcholine occurred in control tissues, the DR in the presence of antagonist was corrected for this change by dividing the DR for tissues in the presence of antagonists by the DR obtained in parallel control tissues. Finally, the log (DR-1) was expressed as a function of log [antagonist], and the pA_2 value was calculated according to Arunlakshana & Schild (1959). The slope of the Schild plot was considered to be not different from unity when the 95% confidence interval for the slope includes 1.0. Then, pK_B values were obtained from plots constrained to a slope of 1.0.

Pearson correlation coefficients (r) and associated P-values were calculated using the program GraphPad Prism, version 3.0, for the relationship of binding affinity data generated at the five human recombinant muscarinic receptors with our potency and affinity data.

Drugs used

Acetylcholine chloride, atropine sulphate, choline chloride, dimethylsulfoxide (DMSO), guanethidine sulphate, indomethacin and L-N^G-nitroarginine methyl ester (L-NAME) were obtained from Sigma (St. Louis, MO, U.S.A.), hemicholinium-3-bromide, p-fluoro-hexahydro-sila-difenidol hydrochloride (p-F-HHSiD), methoctramine 4 hydrochloride and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) were obtained from RBI (Natick, U.S.A.), (11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3b] [1,4]-benzodiazepine-6-one) (AF-DX 116) was obtained from Karl Thomae GmbH (Biberach, Germany), pirenzepine dihydrochloride from Boehringer Ingelheim (Brussels, Belgium), [methyl-^{3}H]-choline chloride (2775 GBq/mmol) from NEN (Boston, U.S.A.), hexamethonium chloride from Federa (Brussels, Belgium) and mamba toxin-3 (MT-3) and tetrodotoxin (TTX) from Alomone labs (Jerusalem, Israel).

Drugs were dissolved and diluted with distilled water, except for 4-DAMP and AF-DX 116 which were dissolved in DMSO before dilution with distilled water. Stock solutions of 10^{-3} M TTX, 10^{-2} M of atropine, pirenzepine, methoctramine and p-F-HHSiD were kept frozen at -20°C and 10^{-6} M MT-3 was kept at 4°C. Dilutions were made the day of the experiment.

Results

General observations

Tissues responded to EFS (40 V, 0.25 ms, 4 Hz, 2 min) in the presence of L-NAME (3×10^{-4} M) with a biphasic contraction. Fast contractions occurred in all tissues stimulated, but the initial contraction was sometimes followed by a small decrease of tone and a more sustained contraction whereof the amplitude was smaller than that of the initial phasic contraction; in other tissues the initial contraction was very shortly stopped before the contraction gradually increased to an amplitude higher than the initial contraction. When ending the electrical stimulation, tone declined as quickly as it had risen. The highest amplitude of the electrically-induced contractions was measured. After 3 to 5 electrical stimulations, the contraction amplitude was stable. Addition of exogenous acetylcholine (range 3×10^{-7} to 10^{-5} M) caused monophasic reproducible contractions.

In control tissues of the release experiments, the release of tritium before S_1 (sample 3) was 660 ± 50 Bq per g tissue ($n=14$). The amount of tritium released due to S_1 (samples

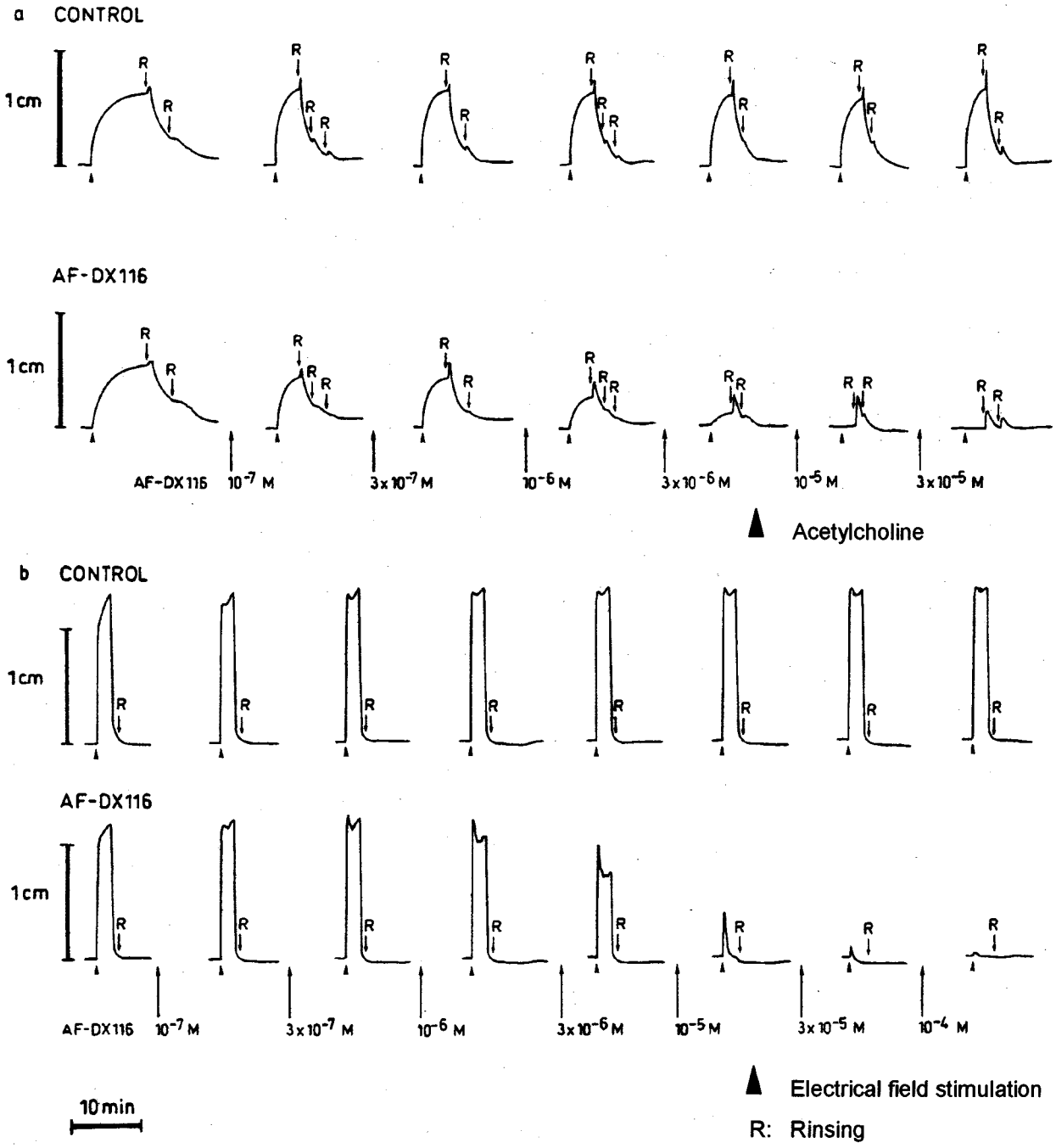

Figure 1 Representative traces showing the contractions to exogenous acetylcholine (3×10^{-7} M; a) and electrical field stimulation (b) in the presence of solvent or increasing concentrations of AF-DX 116. AF-DX 116 was incubated for 30 min before tissues were stimulated.

5, 6, 7 and 8) above calculated basal release (2590 ± 180 Bq per g tissue) was 2700 ± 520 Bq per g tissue ($n = 14$). The outflow evoked by S_2, S_3, S_4 and S_5 was 0.80 ± 0.02, 0.65 ± 0.02, 0.54 ± 0.02 and 0.43 ± 0.02, respectively ($n = 14$) of that caused by S_1. In the experiments with AF-DX 116 and 4-DAMP, where parallel control tissues received dilutions of the solvent DMSO, these values were not significantly different from values of control tissues receiving aqua as solvent.

Effect of muscarinic receptor antagonists on acetylcholine-induced contractions and on electrically-induced tritium outflow and contractions

Contractions induced by a fixed concentration of acetylcholine were not influenced by TTX (3×10^{-6} M) and hexamethonium (5×10^{-4} M), while atropine (10^{-6} M) completely blocked the contractions, indicating that acetylcholine causes contraction by stimulation of postsynaptic muscular mus-

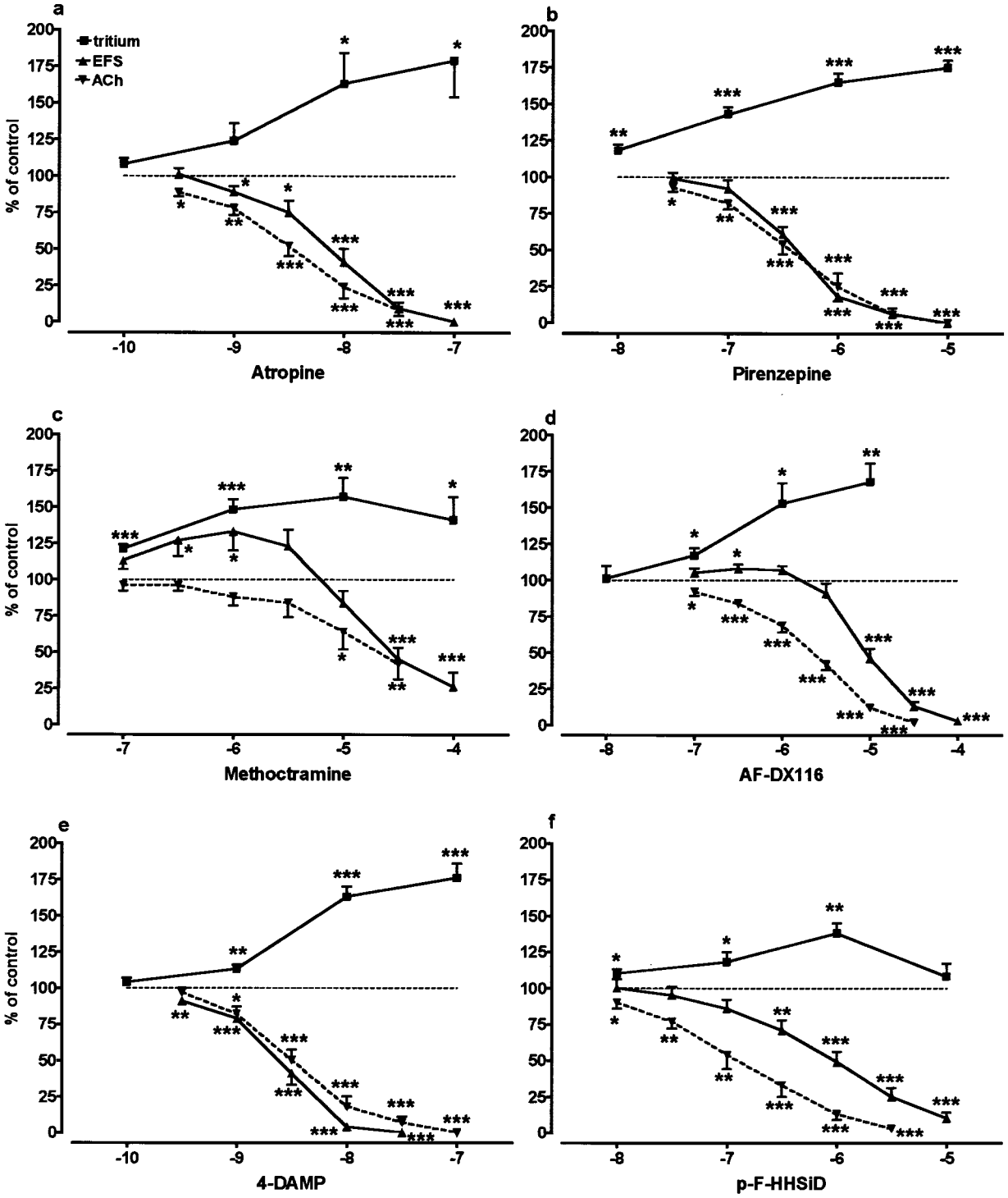

Figure 2 Means ± s.e.mean ($n = 6-8$) of the effect of muscarinic antagonists on tritium outflow (tritium) induced by electrical stimulation and smooth muscle contraction evoked by either electrical field stimulation (EFS) (40 V, 0.25 ms, 4 Hz, 2 min) or exogenous acetylcholine (ACh) in the circular muscle of pig gastric fundus strips. The ratio of the response in the presence of antagonist *versus* the control response before addition of antagonist (S_n/S_1) was expressed as per cent of the same ratio in parallel control tissues receiving the solvent of the antagonist. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ Significantly different from the value in the absence of antagonist (= 100%).

carinic receptors, and thus the effect on the contractions by muscarinic antagonists is solely at the postsynaptic level. As shown in Figures 1a and 2, atropine, pirenzepine, AF-DX 116, 4-DAMP and p-F-HHSiD inhibited the acetylcholine-induced contractions at all concentrations tested, while methoctramine inhibited the acetylcholine-induced contractions only significantly from 10^{-5} M upwards ($n = 6$; $P < 0.05$; Figure 2c). MT-3 (3×10^{-11} to 10^{-8} M) did not influence the acetylcholine-induced contractions ($n = 6$; data not shown). The negative logarithms of the postsynaptic IC_{50} values of

Table 1 Comparison between pre- and postsynaptic potencies (pIC_{50}) of muscarinic antagonists in circular muscle strips of the pig gastric fundus

Antagonist	pIC_{50pre} [a]	*n*	pIC_{50post} [b]	*n*	$pIC_{50(EFS)}$ [c]	*n*
Atropine	8.12±0.18	6	8.48±0.11	8	8.10±0.12+	8
Pirenzepine	7.09±0.05	6	6.37±0.12***	8	6.35±0.06***	8
AF-DX116	6.65±0.32	6	5.69±0.08**	8	5.03±0.08***, +++	8
Methoctramine	6.43±0.14	7	5.19±0.14***	6	4.72±0.07***, ++	8
4-DAMP	8.25±0.04	6	8.48±0.08*	8	8.54±0.07**	8
p-F-HHSiD	7.03±0.30	6	6.85±0.13	8	6.09±0.11**, +++	7

The pIC_{50} values are means±s.e.mean. [a]Assessed *versus* EFS-induced tritium release; [b]Assessed *versus* acetylcholine-induced contraction; and [c]Assessed *versus* EFS-induced contraction. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Significantly different from pIC_{50pre}; +$P<0.05$, ++$P<0.01$, +++$P<0.001$. Significantly different from pIC_{50post}

the antagonists, determined *versus* acetylcholine, are given in Table 1. The rank order of potency of the antagonists at postsynaptic level was: atropine = 4-DAMP > p-F-HHSiD > pirenzepine > AF-DX 116 > methoctramine.

In release studies, the electrically-evoked tritium outflow was abolished by TTX, ω-conotoxin-GVIA or removal of extracellular calcium, and contains a consistent amount of acetylcholine as determined by HPLC (Leclere & Lefebvre, 2001), indicating that electrical stimulation activates cholinergic neurones releasing acetylcholine. This acetylcholine inhibits its own release by activation of presynaptic muscarinic receptors on the cholinergic neurones (Leclere & Lefebvre, 2001), and changes in electrically-induced tritium release by muscarinic antagonists are only related to interference with these presynaptic muscarinic receptors on these cholinergic neurones. None of the muscarinic antagonists affected the basal outflow of tritium, while all antagonists, except MT-3, increased concentration-dependently the electrically-evoked tritium outflow (Figure 2). Methoctramine and p-F-HHSiD showed bell-shaped concentration-response curves. MT-3 (3×10^{-11} to 10^{-8} M) did not influence the electrically-induced tritium release ($n=6$; data not shown). However, higher concentrations of MT-3 decreased the electrically-induced release (10^{-8} M: 101±2%; 3×10^{-8} M: 87±2% ($P<0.01$); 10^{-7} M: 81±3% ($P<0.01$) ($n=6$)). The negative logarithms of the presynaptic IC_{50} values of the antagonists are given in Table 1. The rank order of potency of the antagonists at presynaptic level was: 4-DAMP > atropine > pirenzepine ⩾ p-F-HHSiD > AF-DX 116 > methoctramine. As acetylcholine release from cholinergic nerve endings towards the longitudinal muscle layer might contribute to the tritium measured in the whole tissue strips, a small series of experiments measuring tritium release was performed in the absence of the longitudinal muscle layer and myenteric plexus so that only the tritium release of nerve endings in the circular muscle layer was evaluated ($n=4$; data not shown). The amount of tritium released was to low for obtaining reproducible results.

The electrically-induced contractions were completely blocked by atropine (10^{-6} M) and TTX (3×10^{-6} M), while hexamethonium (5×10^{-4} M) was without effect, indicating the activation of postganglionic cholinergic neurones during EFS. The released acetylcholine causes contraction by stimulating postsynaptic muscular muscarinic receptors but this contraction will be influenced by the effect of the released acetylcholine on its own release by stimulation of presynaptic muscarinic receptors on the cholinergic neurones. Antagonists tested *versus* the electrically-evoked contractions can thus interfere with the response at both pre- and postsynaptic muscarinic receptors. The results are shown in Figure 2 and the pIC_{50} values are given in Table 1. Pirenzepine and 4-DAMP concentration-dependently inhibited the contractions induced by EFS and the concentration-response curve paralleled that *versus* the acetylcholine-induced contractions. In contrast, lower concentrations of methoctramine and AF-

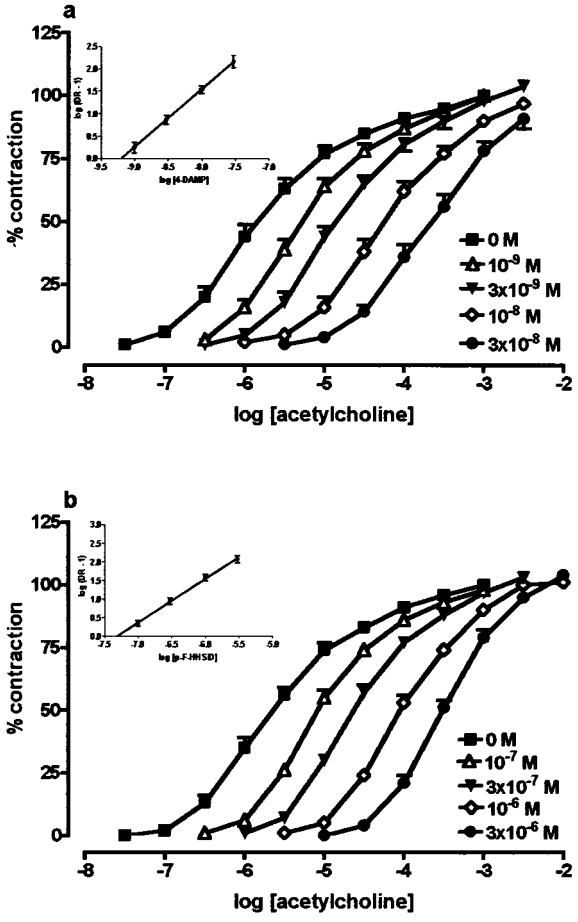

Figure 3 Cumulative concentration-response curves for acetylcholine-induced contractions, expressed as a percentage of the maximum to acetylcholine in the first concentration-response curve, in the circular muscle of pig gastric fundus strips in the absence and presence of different concentrations of (a) 4-DAMP and (b) p-F-HHSiD. Inserts show the Schild plots of the respective antagonists. The pA_2 values and slopes are given in Table 2. Data points represent means±s.e.mean of eight independent experiments.

DX 116 (Figure 1b) increased the amplitude of the electrically-induced contractions, while higher concentrations decreased it. Although atropine and p-F-HHSiD did not enhance the electrically-induced contractions, the concentration-response curve *versus* these contractions was moderately (atropine) to markedly (p-F-HHSiD) shifted to the right in comparison with that *versus* acetylcholine-induced contractions. MT-3 (3×10^{-11} to 10^{-8} M) did not influence the electrically-induced contractions ($n=6$; data not shown). However, higher concentrations tended to increase the electrically-induced contractions (10^{-8} M: $100\pm4\%$; 3×10^{-8} M: $101\pm6\%$; 10^{-7} M: $108\pm7\%$ ($n=4$)).

The effect of pirenzepine was also studied on electrically- and acetylcholine-induced contractions in strips cut in the longitudinal direction so that the contractions were due to longitudinal smooth muscle activity. In these tissues, the pIC_{50} value of pirenzepine *versus* electrically-induced contractions (6.55 ± 0.13; $n=4$) was significantly larger than that *versus* acetylcholine-induced contractions (6.06 ± 0.12; $n=4$; $P<0.05$).

Postsynaptic affinities of muscarinic receptor antagonists versus *acetylcholine*

Acetylcholine caused concentration-dependent contractions with an EC_{50} of $2.05\pm0.41\times10^{-6}$ M ($n=40$), and the maximal contraction was $111\pm1\%$ as percentage of the KCl (80 mM)-induced contraction ($n=40$). Except for MT-3 (3×10^{-10} to 10^{-8} M; $n=2$), parallel rightward shifts of the concentration-response curves to acetylcholine, without depression of the maximum response, were obtained with the muscarinic antagonists (atropine: 3×10^{-9} to 10^{-7} M, $n=6$; pirenzepine: 10^{-7} to 3×10^{-6} M, $n=6$; AF-DX 116: 10^{-6} to 3×10^{-5} M, $n=6$; methoctramine: 3×10^{-6} to 3×10^{-5} M, $n=8$; 4-DAMP: 10^{-9} to 3×10^{-8} M, $n=8$ (Figure 3a); p-F-HHSiD: 10^{-7} to 3×10^{-6} M, $n=8$ (Figure 3b)). In two out of eight tissues, methoctramine (3×10^{-6} to 3×10^{-5} M) produced no significant parallel rightward shift of the concentration-response curve. The Schild plot was therefore performed on the basis of the results in the six tissues where methoctramine was active. Schild regression analysis was linear with a slope not significantly different from unity for atropine, pirenzepine, 4-DAMP and p-F-HHSiD (Table 2). The rank order of antagonist affinities was 4-DAMP > atropine > p-F-HHSiD > pirenzepine > AF-DX 116 > methoctramine.

MT-3 (10^{-7} M; $n=4$) caused a parallel leftward shift of the concentration-response curve to acetylcholine, without depression of the maximum response. The pEC_{50} of acetylcholine in the control strips was 5.54 ± 0.19, while this was 6.25 ± 0.18 in the presence of MT-3 ($P<0.05$). However, MT-3 (10^{-7} M; $n=4$) had no effect on the concentration-response curve to KCl. The pEC_{50} of the control strips was 1.67 ± 0.04, while this was 1.75 ± 0.02 in the presence of MT-3.

Table 2 Postsynaptic affinity values of muscarinic antagonists at receptors mediating contraction of circular muscle strips of the pig gastric fundus in response to acetylcholine

Antagonist	pA_2	*Slope*	pK_B	n
Atropine	8.94 ± 0.07	0.87 ± 0.07	8.79 ± 0.04	6
Pirenzepine	6.76 ± 0.19	0.98 ± 0.15	6.70 ± 0.09	6
AF-DX 116	5.96 ± 0.12	0.71 ± 0.07		6
Methoctramine	5.55 ± 0.25	0.20 ± 0.09		6
4-DAMP	9.09 ± 0.16	1.05 ± 0.09	9.13 ± 0.05	8
p-F-HHSiD	7.13 ± 0.13	0.94 ± 0.12	7.01 ± 0.08	8

Values are means ± s.e.mean. The antagonist action of each antagonist was estimated by Schild plot analysis, and pA_2 values and the slope of the linear regression were calculated. When the 95% confidence limits of the slope included the value 1.0, the line was considered to be not significantly different from unity, and pK_B values were obtained from plots constrained to a slope of 1.0.

Discussion

In this study we describe the pharmacological profile of muscarinic receptor(s) involved in the control of acetylcholine release and smooth muscle contraction of the circular muscle of the pig gastric fundus. Because of the lack of selective muscarinic antagonists, a series of subtype-preferring antagonists are used to characterize pharmacologically the involvement of a particular muscarinic receptor. The muscarinic antagonists used in this study to determine the profile of the pre- and postsynaptic muscarinic receptors of the circular muscle of the pig gastric fundus are the non-selective antagonist atropine, the M_1-preferring antagonist pirenzepine, the M_2-preferring antagonists AF-DX 116 and methoctramine, the M_3-preferring antagonists 4-DAMP and p-F-HHSiD and the M_4-preferring antagonist MT-3.

As described in the Results section, the postsynaptic muscarinic receptors are assessed when studying the influence of the muscarinic antagonists on acetylcholine-induced contractions. The tissues are mounted in the direction of the circular muscle layer to measure these contractions and the short parts of longitudinal muscle in the tissues will not contribute to the contractions; this series of experiments thus evaluates the postsynaptic muscarinic receptors on the circular muscle cells of the pig gastric fundus. The presynaptic muscarinic receptors are assessed when studying the influence of the muscarinic antagonists on electrically-induced tritium release; cholinergic neurones directed to the longitudinal

Table 3 Affinity values (pK_i) at muscarinic receptors from literature

	M_1	M_2	M_3	M_4	M_5
Atropine	9.27	8.96	9.39	9.11	9.11
Pirenzepine	7.96	6.24	6.82	7.11	6.73
AF-DX 116	6.44	7.20	6.07	6.68	5.29
Methoctramine	7.08	7.78	6.40	6.89	6.36
4-DAMP	9.03	8.14	9.28	8.49	8.91
p-F-HHSiD	7.30	6.41	7.56	7.21	6.73
MT-3	6.78	<6.3	6.3	8.33	

Affinity values (pK_i) are the mean values which refer to radioligand binding studies at cortex (M_1), heart (M_2), submandibular and lacrimal gland (M_3) and human cloned muscarinic receptors expressed in Chinese hamster ovary (CHO) cells (data from Lazareno & Roberts, 1989; Lazareno *et al*., 1990; Pedder *et al*., 1991; Doods *et al*., 1993; Esqueda *et al*., 1996; Hegde *et al*., 1997). For MT-3, affinity values (pA_2) refer to the effect of MT-3 on acetylcholine stimulation of [^{35}S]GTPγS binding to membranes of CHO cells expressing the cloned human M_1-M_4 receptors (Olianas *et al*., 1999).

muscle might contribute to the tritium release in this series, and interference of the muscarinic antagonists with presynaptic muscarinic receptors on these neurones might influence the results. The release experiments were performed in the absence of the acetylcholinesterase inhibitor physostigmine. We indeed previously demonstrated (Leclere & Lefebvre, 2001) that the S_n/S_1 ratio for tritium is systematically similar to that for [^{3}H]-acetylcholine, so it is not necessary to separate the radioactive components, which requires the presence of physostigmine. Muscarinic antagonists can interfere with both pre- and postsynaptic muscarinic receptors when studied *versus* electrically-induced contractions, as illustrated by the results with atropine. The pIC_{50} of atropine *versus* electrically-induced contractions was significantly lower than the pIC_{50} *versus* acetylcholine-induced contractions; the stimulatory effect on acetylcholine release by antagonism of the presynaptic muscarinic receptors will indeed counteract the antagonistic effect *versus* acetylcholine at the postsynaptic level. As the measured item is contraction, the presynaptic muscarinic receptors on the cholinergic neurones to the longitudinal muscle will not interfere in this assay.

Characterization of the postsynaptic muscarinic receptors

The rank order of potencies (Table 1) and affinities (Table 2) at the postsynaptic level of the six investigated subtype-preferring antagonists is similar, and comparing these rank orders with the rank order of the binding constants for the five muscarinic receptor subtypes (Table 3) is consistent with the pharmacological profile of the M_3 and M_5 receptor subtypes. However, analysis of the relationship of our potency and affinity values with literature data of binding affinity values for the muscarinic receptor subtypes shows that the best correlation coefficient (potency: M_3: $r=0.977$, $P=0.001$; M_5: $r=0.930$, $P=0.007$ and affinity: M_3: $r=0.977$, $P=0.001$; M_5: $r=0.941$, $P=0.005$) was found for the M_3 subtype. Our conclusion is similar to findings in gastrointestinal smooth muscle preparations (e.a. Lazareno & Roberts, 1989; Doods *et al*., 1994; Preiksaitis & Laurier, 1998; Shi & Sarna, 1997, 1999) and in other tissues (see review Eglen *et al*., 1996), where the postsynaptic muscarinic receptors also belong to the M_3 subtype. The slope of the Schild plot for AF-DX 116 and especially for methoctramine was very small. In the guinea-pig LMMP preparations, the slope for methoctramine *versus* acetylcholine-induced contractions was also significantly less than unity (Barocelli *et al*., 1994). One possible explanation might be the antagonism of a heterogenous receptor population (Kenakin, 1993). Indeed, many investigators demonstrated the presence of M_2 receptors together with M_3 receptors on smooth muscle *via* molecular and radioligand binding studies, or pharmacologically *via* indirect methods (see reviews Ehlert *et al*., 1999; Eglen, 2001). Although the majority of the muscular muscarinic receptors belong to the M_2 subtype, muscarinic agonists mainly cause contraction *via* stimulation of the M_3 receptors. Stimulation of M_2 receptors inhibits adenylate cyclase, and they will oppose the relaxations due to activation of adenylate cyclase by e.g. stimulation of β-adrenoceptors (Ehlert *et al*., 1999; Eglen, 2001). The M_2 receptors might also have an important effect during inflammation (Shi & Sarna, 1997, 1999). However, when using the method described by Hegde *et al*. (1997) to determine the presence of these M_2 receptors, no evidence was found for the presence of M_2 receptors on the pig gastric fundus (results not shown).

Characterization of the presynaptic muscarinic receptors

The results of the experiments performed to characterize the presynaptic muscarinic receptors do not allow to strongly support one particular subtype.

The rank order of potencies of the muscarinic antagonists at the presynaptic level, as assessed *via* their influence on tritium release (Table 1) corresponds with the pharmacological profile of the M_1 subtype (Table 3) and the correlation between the presynaptic pIC_{50} values (Table 1) with published binding affinity values for the M_1 receptor (Table 3) is significant ($r=0.941$, $P=0.005$), but other points argue against M_1 receptors. First, the potency of pirenzepine was almost 10 fold lower than expected at M_1 receptors (see Table 3). Second, pirenzepine did not discriminate between the electrically- and acetylcholine-induced contractions, suggesting that the auto-receptor is not an M_1 receptor. Nevertheless, the pIC_{50} value for pirenzepine obtained *versus* acetylcholine-induced contractions was significantly lower than that on tritium outflow. This might be due to the presence of inhibitory M_1 receptors on cholinergic neurones innervating the longitudinal smooth muscle of the pig gastric fundus and contributing to the higher potency of pirenzepine on tritium outflow. Indeed, a different type of presynaptic muscarinic receptors has already been demonstrated on nerve endings in the circular *versus* longitudinal smooth muscle of the guinea-pig ileum (Soejima *et al*., 1993; Dietrich & Kilbinger, 1995). Release experiments in circular muscle strips without longitudinal muscle layer and myenteric plexus, to avoid the possible interference from the cholinergic nerve endings in the longitudinal muscle, did not yield reproducible results. Therefore, pirenzepine was tested in strips cut in the direction of the longitudinal muscle layer. The pIC_{50} value of pirenzepine *versus* electrically-induced contractions was larger than those *versus* acetylcholine-induced contractions in these strips, suggesting that facilitatory M_1 receptors are present on the cholinergic neurones innervating the longitudinal muscle. We have thus no explanation for the results with pirenzepine in the circular muscle strips.

When looking at the pIC_{50} values (Table 1), the M_2 subtype preferring muscarinic antagonists AF-DX 116 and methoctramine were more potent in facilitating the evoked tritium release than in inhibiting the contractile response. Also, electrically-evoked contractions were enhanced at the lowest concentrations, presumably due to facilitation of acetylcholine release. These results would correlate with the presence of presynaptic M_2 receptors, but this possibility cannot be maintained. Indeed (1) The rank order of the pIC_{50} values *versus* electrically-induced tritium release did not correspond with that of an M_2 receptor; and (2) When comparing the presynaptic pIC_{50} values with published pK_i values for the M_2 muscarinic subtype ($r=0.581$, $P=0.227$), no significant correlation was found.

With regard to M_4 receptors, a significant correlation was found between our presynaptic pIC_{50} values and the pK_i values from literature ($r=0.944$, $P=0.005$). However, it is not possible to define with certainty that the presynaptic muscarinic receptor belongs to the M_4 subtype. Indeed, the M_4 antagonist MT-3 had no effect on the electrically-induced

contractions and tritium release up to 10^{-8} M. As the pK_i value of MT-3 on M_4 receptors is 8.33 (Olianas *et al*., 1999), some effect of 10^{-8} M can be expected. Indeed, D'Agostino *et al*. (2000) demonstrated that in the human detrusor MT-3 increases [^{3}H]-acetylcholine release by acting at the M_4 receptor with a pIC_{50} value of 8.50, also illustrating that the substance penetrates in muscle strips. Higher concentrations of MT-3 (3×10^{-8} to 10^{-7} M) even decreased the electrically-induced tritium release in our study. This would correlate with the presence of facilitatory M_4 receptors rather than inhibitory M_4 receptors on the cholinergic neurones. However, one might then also expect a decrease in the electrically-induced tritium release at higher concentrations of atropine, pirenzepine, AF-DX 116 and 4-DAMP in view of their capacity at M_4 receptors (see Table 3). Another possibility is that MT-3, which is a toxin (Adem & Karlsson, 1997; Jerusalinsky *et al*., 2000), has a toxic effect at these concentrations. Remarkably, although the electrically-induced release decreased at these high concentrations of MT-3, the electrically-induced contractions tended to increase. This is probably due to a postsynaptic effect of MT-3 whereby MT-3 interferes with the signal transduction of acetylcholine. Indeed, MT-3 caused a leftward shift of the concentration-response curve to acetylcholine, while it did not influence the concentration-response curve to KCl.

With regard to M_3 receptors, the presynaptic pIC_{50} values of the antagonists used correlated closely with the average of the binding affinities for the M_3 receptor ($r=0.965$, $P=0.002$), suggesting the presence of presynaptic M_3 receptors, inhibiting the release of acetylcholine. This supports findings in the guinea-pig and canine LMMP preparations, where presynaptic M_3 receptors were responsible for the inhibition of acetylcholine release (Kostka *et al*., 1989; Soejima *et al*., 1993). However, it should be noted that a good correlation was also obtained with the average of binding affinity values for the cloned human M_5 receptor ($r=0.945$, $P=0.005$). This may not surprise given the fact that most antagonists discriminate poorly between M_3 and M_5 receptors. It can also not be excluded that more than one subtype of muscarinic receptor is involved in the presynaptic inhibitory control of acetylcholine release.

In conclusion, the postsynaptic contractile muscarinic receptors in the circular muscle of the pig gastric fundus seem to belong to the M_3 receptor subtype, while the presynaptic muscarinic receptor cannot be clearly defined.

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