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Novel pharmacological profile of muscarinic receptors mediating contraction of the guinea-pig uterus*

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Summary. The present study was designed to further characterize the muscarinic receptors mediating contraction of the guinea-pig uterus. The affinities of various selective muscarinic antagonists were determined and compared with those obtained at M_1 (rabbit vas deferens), M_2 (guinea-pig atria) and M_3 receptors (guinea-pig ileum).

The contractile responses of uterine smooth muscle from immature guinea-pigs to carbachol ($pD_2 = 5.73$) were competitively antagonized by pirenzepine ($pA_2 = 7.04$), AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one) ($pA_2 = 6.96$), himbacine ($pA_2 = 7.92$), methoctramine ($pA_2 = 7.52$), 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) ($pA_2 = 8.87$) and sila-hexocyclium ($pA_2 = 8.81$). A comparison of affinity values indicates that the muscarinic receptors present in guinea-pig uterus display a novel pharmacological profile which is not consistent with the presence of either an M_1, M_2 or M_3 receptor. The affinities determined for the different antagonists rather showed a close similarity to those obtained at muscarinic receptors present in rat striatum and NG108-15 cells which are considered pharmacological equivalents (M_4 receptors) of the m4 gene product. We thus hypothesize that the guinea-pig isolated uterus preparation may serve as a simple functional assay system to study the pharmacology of M_4 receptors.

Key words: Muscarinic receptors – M_4 receptors – Guinea-pig uterus – Pirenzepine – Methoctramine – Sila-hexocyclium

Introduction

Radioligand binding as well as in vitro and in vivo functional studies have revealed the existence of at least three pharmacologically distinct muscarinic receptor subtypes, which have been termed M_1, M_2 and M_3 (Doods et al. 1987; Mitchelson 1988; Levine and Birdsall 1989). M_1 receptors are mainly present in neuronal tissues (e.g. autonomic ganglia, cerebral cortex and hippocampus) and are characterized by a high affinity for pirenzepine (affinity profile $M_1 > M_3 \geq M_2$) (Hammer et al. 1980; Hammer and Giachetti 1982). M_2 and M_3 receptors are primarily found in lower brain areas and in peripheral effector organs such as heart (M_2), smooth muscle (M_3) and glands (M_3) and display low affinity for pirenzepine. They can be distinguished by the use of selective antagonists such as methoctramine and AF-DX 116 ($M_2 > M_1 > M_3$) (Melchiorre et al. 1987; Waelbroeck et al. 1989; Hammer et al. 1986; Micheletti et al. 1987), himbacine ($M_1 \approx M_2 > M_3$) (Gilani and Cobbin 1986; Lazareno and Roberts 1989), sila-hexocyclium ($M_1 \geq M_3 > M_2$) (Eltze et al. 1988; Waelbroeck et al. 1989), 4-DAMP and hexahydro-sila-difenidol ($M_3 \geq M_1 > M_2$) (Barlow et al. 1976; Mutschler and Lambrecht 1984; Lambrecht et al. 1989).

The pharmacology of the M_1, M_2 and M_3 receptors closely corresponds to that of the m1, m2 and m3 receptor subtypes, respectively, identified by recent receptor cloning studies (Akiba et al. 1988; Buckley et al. 1989). Recombinant DNA technology has also revealed the existence of two additional muscarinic receptor subtypes, m4 and m5, whose pharmacology has not been studied in detail so far (Bonner et al. 1987, 1988; Peralta et al. 1987).

Recently, Eglen et al. (1989) suggested that the contractile response to carbachol in the guinea-pig estrogen-dominated uterus is mediated by a homogenous population of M_2 receptors. As smooth muscle contraction is usually mediated by M_3 receptors (Mitchelson 1988), we have reevaluated this finding using a larger number of selective antagonists. The affinity values obtained in uteri from immature guinea-pigs were compared to those

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obtained at M_1 receptors in rabbit vas deferens, M_2 receptors in guinea-pig atria and smooth muscle M_3 receptors in guinea-pig ileum. Our data are not consistent with the presence of an M_1, M_2 or M_3 receptor in the guinea-pig uterus but suggest that the pharmacology of the uterine muscarinic receptor is most closely related to that present in NG108-15 cells (Michel et al. 1989) and rat striatum (McKinney et al. 1989; Waelbroeck et al. 1990), which is considered the pharmacological correlate (M_4 receptor) of the m4 gene product.

Methods

Guinea-pig isolated uterus. Immature guinea-pigs (Pirbright-white, 190–220 g, 3–4 weeks old) were killed by a blow on the head and uterine horns were excised and separated from fat deposits and mesenteric attachments. Uterine horns were suspended under 1.0 g tension in 7 ml tissue baths containing oxygenated (95% O_2 + 5% CO_2) Sund's physiological salt solution (mmol/l: NaCl 154.0, KCl 5.63, $CaCl_2$ 0.54, $MgCl_2$ 0.95, $NaHCO_3$ 5.95, (+)-glucose 2.78). The preparations were maintained at 30°C and after 1 h equilibration period, isotonic contractions to carbachol were recorded by a force-displacement transducer connected to a Hellige amplifier and a Rikadenki multichannel recorder. Initially, the tissues were exposed to a single concentration of carbachol (3 μmol/l) to check the responsiveness to the agonist. Then two cumulative concentration-response curves to carbachol were constructed, one in the absence and one in the presence of antagonist. In separate control experiments, no significant changes in tissue sensitivity to the agonist was observed over the period necessary for the determination of two concentration-response curves.

Rabbit isolated vas deferens. Experiments on rabbit isolated vas deferens were performed according to Eltze (1988). Male New Zealand white rabbits (2.5–3.0 kg) were killed by i.v. injection of 120 mg/kg pentobarbitone sodium. Vasa deferentia were excised and divided into four segments of approximately 1.5 cm length. The preparations were set up in 7 ml organ baths containing modified Krebs buffer which consisted of (mmol/l): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and (+)-glucose 11.1; 1 μmol/l yohimbine was included to block α_2-adrenoceptors. The bathing fluid was maintained at 31°C and aerated with 95% O_2 + 5% CO_2. A basal tension of 750 mg was applied. After a 30 min equilibration period twitch contractions were elicited by field stimulation (0.05 Hz, 0.5 ms, 30 V) with platinum electrodes and recorded as described for the isolated uterus. The neurogenic twitch contractions were concentration-dependently inhibited by the M_1 receptor agonist 4-Cl-McN-A-343 [4-(4-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide] (Eltze et al. 1988). After 1 h equilibration period, concentration-response curves were constructed by adding doses of 4-Cl-McN-A-343 cumulatively. When these responses were constant, concentration-response curves were repeated in the presence of antagonists.

Guinea-pig isolated atria and ileum. Guinea-pigs (Pirbright-white, 300–400 g) of either sex were killed by cervical dislocation. Left atria and ileal longitudinal muscle strips (Paton and Zar 1968) were set up in 6 ml organ baths, under 500 mg tension, in oxygenated (95% O_2 + 5% CO_2) Tyrode solution (32°C) composed of (mmol/l): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42 and (+)-glucose 5.6. Arecaidine propargyl ester (Moser et al. 1989) was used as an agonist. Atria were paced electrically (2 Hz, 3 ms, 5 V) by means of platinum electrodes. Negative inotropic effects to the agonist were measured as changes in isometric tension. Responses of ileal longitudinal muscle to arecaidine propargyl ester were measured as isotonic contractions. Atrial and ileal responses were recorded as with the isolated uterus. After an 1 h equilibration period, concentration-response curves to arecaidine propargyl ester were obtained by use of cumulative dosing. When two control curves were superimposable, cumulative concentration-response curves were repeated in the presence of antagonists.

Antagonist affinities. Three to four different concentrations of each antagonist (log conc. intervals = 0.5) were used. Antagonists were allowed to equilibrate for 15–30 min (ileum and uterus), 30–45 min (atria) or 30–60 min in vas deferens, respectively. Preliminary experiments indicated that these intervals were sufficient for equilibration of the antagonist concentrations used. The uterus preparation was exposed to only one concentration of antagonist. EC_{50}-values of agonists in the absence and presence of antagonists were determined graphically for calculation of dose-ratios. The slopes of Arunlakshana-Schild plots (Arunlakshana and Schild 1959) were determined by linear regression by the method of least squares. pA_2-values were estimated as the intercept on the abscissa scale by fitting to the data the best straight line with a slope of unity (Tallarida and Murray 1986).

Statistical evaluation. The data are presented as means ± SEM of n experiments. Differences between mean values were tested for statistical significance by Student's t-test; $p < 0.05$ was accepted as being significant.

Drugs. The following drugs were used: carbamoylcholine chloride, tetrodotoxin and yohimbine hydrochloride (Sigma, München, FRG); hexamethonium bromide (Fluka, Buchs, Switzerland); pirenzepine dihydrochloride and AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one) (Thomae, Biberach, FRG); himbacine hydrochloride (kindly donated by Dr. W. C. Taylor, Sydney, Australia); methoctramine tetrahydrochloride (generous gift from Dr. C. Melchiorre, Bologna, Italy); 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) (kindly donated by Dr. R. B. Barlow, Bristol, UK); sila-hexocyclium methyl sulfate (Tacke et al. 1989), 4-(4-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A343) (Nelson et al. 1976) and arecaidine propargyl ester (Mutschler and Hultzsch 1973) were synthesized in our laboratories. All other chemicals were of reagent grade and used as purchased.

Results

The contractile responses of the isolated guinea-pig uterus to carbachol were not significantly ($P > 0.05$) affected by the presence of tetrodotoxin (1 μmol/l) or hexamethonium (10 μmol/l), excluding the involvement of neuronal nicotinic or muscarinic receptors (data not shown).

All antagonists employed concentration-dependently inhibited the carbachol-induced contractile responses of the isolated guinea-pig uterus ($-\log EC_{50} = 5.73 \pm 0.03$) (Fig. 1) as well as the negative inotropic responses in guinea-pig atria ($-\log EC_{50} = 8.12 \pm 0.07$) and ileal contractions ($-\log EC_{50} = 7.77 \pm 0.04$) to arecaidine propargyl ester. Likewise, the 4-Cl-McN-A-343-induced inhibition of twitch contractions of the rabbit vas deferens ($-\log EC_{50} = 7.12 \pm 0.06$) was concentration-dependently blocked by all antagonists examined. In all tissues, parallel shifts of the agonist concentration-response curves without any appreciable changes of basal tension or reduction of maximum responses were observed. This is shown for pirenzepine, methoctramine and

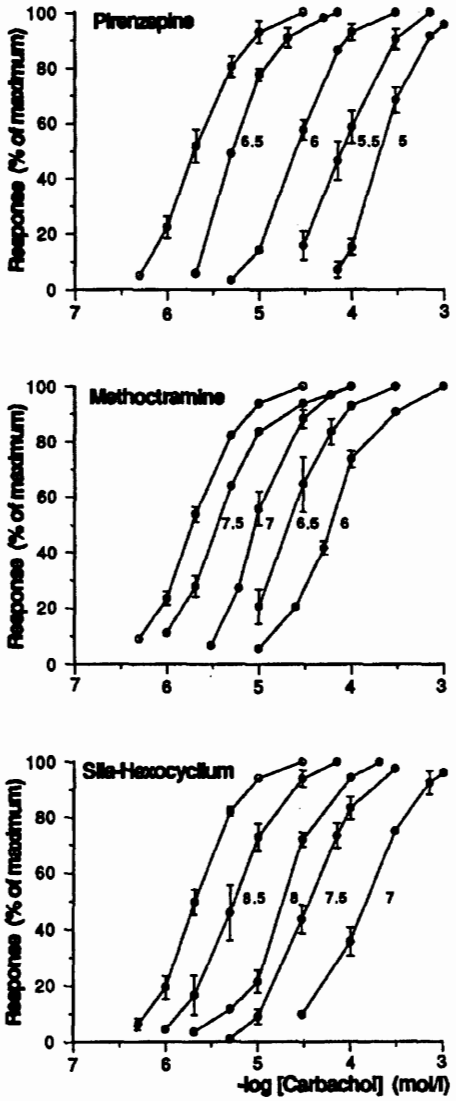

Fig. 1. Cumulative concentration-response curves for carbachol-induced contractions of the guinea-pig uterus in the absence (○) and presence (●) of increasing concentrations of pirenzepine, methoctramine and sila-hexocyclium. Numbers next to the concentration-response curves indicate −log molar antagonist concentrations. Contractile responses to each concentration of agonist are expressed as a percentage of the maximum contraction induced by carbachol. The data points represent mean of $n = 3-5$ observations (SEM are given as *vertical bars* if larger than symbol size)

sila-hexocyclium in Fig. 1. Arunlakshana-Schild plots were linear through the concentration range tested for each antagonist and slopes were not significantly different from unity ($p > 0.05$) (Table 1). Thus, all compounds behaved as competitive muscarinic antagonists in all preparations used. The pA_2-values determined for the various antagonists are shown in Table 1. As indicated, some data are taken from work previously published (Lambrecht et al. 1989; Waelbroeck et al. 1989). A comparison of pA_2-values reveals that the antagonist affinities obtained at uterine muscarinic receptors of the guinea-pig do not correlate well with those determined at M_1, M_2 or M_3 receptors (Table 1, Fig. 2A–C).

Discussion

Receptor binding studies suggest that smooth muscle of the ileum (Giraldo et al. 1988), urinary bladder (Monferini et al. 1988) and trachea (Roffel et al. 1989) have both M_2 and M_3 receptors. Functionally, however, smooth muscle contraction appears to be mediated through M_3 receptors only (Mitchelson 1988). In contrast to these reports, Eglen et al. (1989) suggested that the muscarinic receptor mediating smooth muscle contraction of the guinea-pig uterus is of the M_2 type. We have reevaluated this controversial finding using a larger number of selective antagonists. The affinities obtained at uterine muscarinic receptors of immature guinea-pigs were compared to those determined at M_1 (rabbit vas deferens), M_2 (guinea-pig atria) and M_3 receptors (guinea-pig ileum).

The antimuscarinic potencies of the antagonists at M_1, M_2 and M_3 receptors (pA_2-values, Table 1) were in good agreement with their affinity estimates determined in radioligand binding studies (Lazareno and Roberts 1989; Waelbroeck et al. 1989) and previous functional studies (Barlow et al. 1976; Gilani and Cobbin 1986; Micheletti et al. 1987; Eltze 1988; Eltze et al. 1988; Waelbroeck et al. 1989; Lambrecht et al. 1989). A comparison of these affinity values with those determined at uterine muscarinic receptors suggests that the muscarinic receptor mediating contractions of the guinea-pig uterus is pharmacologically unique (Table 1). As discussed below, its profile is not consistent with the presence of an M_1, M_2 or M_3 receptor in this tissue. Moreover, since the slopes of Arunlakshana-Schild regression lines did not significantly differ from unity, the presence of a heterogeneous mixture of these subtypes appears to be unlikely (Table 1). This conclusion is further supported by radioligand binding studies which have identified a homogeneous population of muscarinic binding sites in the guinea-pig uterus (Eglen et al. 1989).

The affinity of pirenzepine ($pA_2 = 7.04$) for uterine muscarinic receptors was 16-fold lower than that observed at M_1 receptors in vas deferens, excluding the presence of M_1 receptors (Table 1, Fig. 2A). Furthermore, uterine muscarinic receptors displayed high affinity for AF-DX 116 ($pA_2 = 6.96$), himbacine ($pA_2 = 7.92$) and methoctramine ($pA_2 = 7.52$). Their pA_2-values closely approached those obtained in guinea-pig atria (Fig. 2B) but were 3-, 4- and 24-fold higher, respectively, than observed at ileal M_3 receptors (Fig. 2C). These data, in conjunction with the low affinity found for pirenzepine, would indicate a high similarity of the uterine receptors to cardiac M_2 receptors. However, the high antimuscarinic potencies of the selective antagonists 4-DAMP ($pA_2 = 8.87$) and sila-hexocyclium ($pA_2 = 8.81$) do not fit into this concept. These antagonists show a 3- and 17-fold higher affinity for muscarinic receptors in the uterus than for atrial M_2 receptors (Fig. 2B).

Table 1. pA_2-values and slopes of Arunlakshana-Schild plots (in parentheses) for various selective muscarinic antagonists at M_1, M_2, M_3 and guinea-pig uterine muscarinic receptors

Compound	Rabbit vas deferens (M_1)	Guinea-pig atria (M_2)	Guinea-pig ileum (M_3)	Guinea-pig uterus
Pirenzepine	8.24 ± 0.06* (1.19 ± 0.10)	6.82 ± 0.03* (0.98 ± 0.02)	6.88 ± 0.04* (1.07 ± 0.04)	7.04 ± 0.02 (1.00 ± 0.03) [$n = 14$; 14]
AF-DX 116	7.12 ± 0.04 (1.07 ± 0.08) [$n = 8$; 4]	7.36 ± 0.05 (0.89 ± 0.08) [$n = 8$; 8]	6.48 ± 0.04 (0.93 ± 0.07) [$n = 10$; 9]	6.96 ± 0.03 (0.92 ± 0.05) [$n = 12$; 12]
Himbacine	8.17 ± 0.04 (1.02 ± 0.06) [$n = 13$; 7]	8.09 ± 0.04 (0.86 ± 0.07) [$n = 8$; 8]	7.34 ± 0.05 (0.93 ± 0.08) [$n = 8$; 4]	7.92 ± 0.03 (0.99 ± 0.05) [$n = 19$; 19]
Methoctramine	6.85 ± 0.07* (1.19 ± 0.10)	7.69 ± 0.03* (1.05 ± 0.05)	6.17 ± 0.05* (0.99 ± 0.09)	7.51 ± 0.03 (0.97 ± 0.04) [$n = 15$; 15]
4-DAMP	9.39 ± 0.06 (1.09 ± 0.12) [$n = 8$; 6]	8.40 ± 0.05 (0.93 ± 0.08) [$n = 8$; 8]	9.34 ± 0.05 (0.91 ± 0.05) [$n = 8$; 8]	8.87 ± 0.01 (1.01 ± 0.02) [$n = 18$; 18]
Sila-Hexocyclium	9.04 ± 0.03 (1.07 ± 0.06) [$n = 8$; 6]	7.57 ± 0.04** (0.94 ± 0.07)	8.78 ± 0.05** (1.04 ± 0.09)	8.81 ± 0.03 (0.97 ± 0.05) [$n = 16$; 16]

The data are presented as means $\pm$ SEM. The slopes of Arunlakshana-Schild plots were calculated by linear regression analysis (Tallarida and Murray 1986). pA_2-values were obtained after the unity constraint had been imposed. The number of total data points (n) and tissues used are given in square brackets. The slopes shown are not significantly different from unity ($p > 0.05$). * Data taken from Lambrecht et al. 1989; ** Data previously published (Waelbroeck et al. 1989)

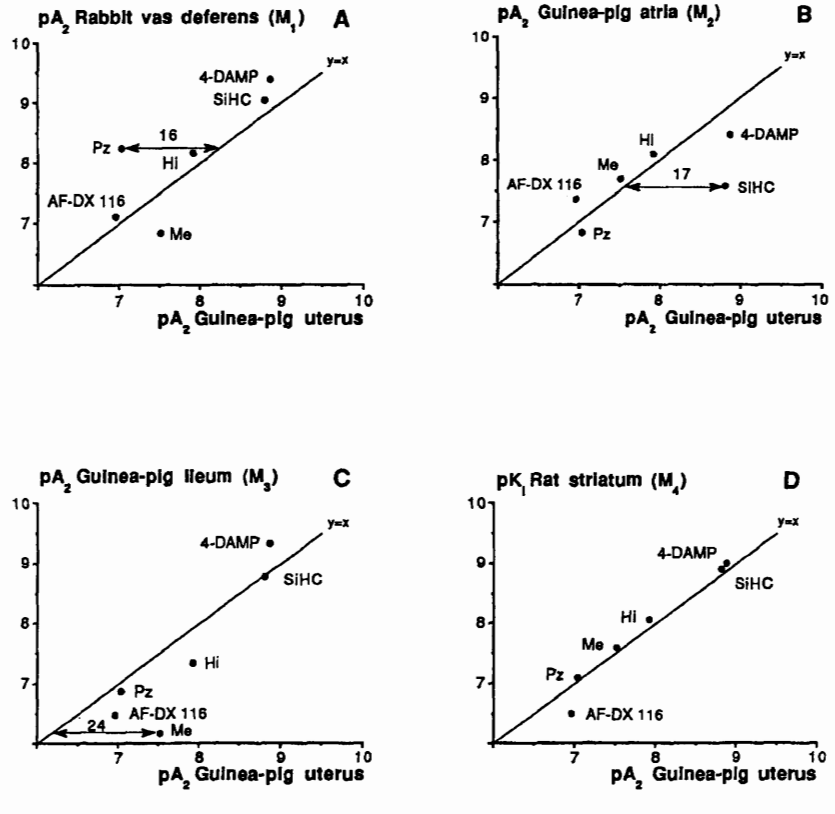

Fig. 2A–D. Comparison of mean antagonist affinities (pA_2-values, Table 1) obtained for pirenzepine (Pz), AF-DX 116, himbacine (Hi), methoctramine (Me), 4-DAMP and sila-hexocyclium (Sihc) at muscarinic receptors in guinea-pig uterus to their affinity estimates determined at M_1 (**A**), M_2 (**B**) and M_3 (**C**) receptors. Comparison of mean antagonist affinities (pA_2- or pK_i-values) obtained in functional studies in guinea-pig uterus and radioligand binding assays at M_4 receptors present in rat striatum (Waelbroeck et al. 1990), (**D**). In case of receptor identity, the data points should not significantly deviate from the theoretical equality line ($y = x$). The distance to this line (given in Fig. 2A–C for pirenzepine, sila-hexocyclium and methoctramine as the antilog of the difference in pA_2-values) is a direct measure of receptor selectivity

Taken together, the muscarinic receptors present in guinea-pig uterus display a novel pharmacological profile whose most characteristic features are a low affinity for pirenzepine but high affinity for methoctramine and silahexocyclium.

Recent findings suggest that the muscarinic receptors in rat striatum (McKinney et al. 1989; Waelbroeck et al. 1990) and NG108-15 cells (Michel et al. 1989; Baumgold and White 1989) are pharmacological equivalents (M_4 receptors) of the m4 gene (Brann et al. 1988; Peralta et al. 1987). In Fig. 2D, antagonist affinities (pA_2-values) for muscarinic receptors in guinea-pig uterus are plotted against their binding affinities (pK_i-values) for M_4 receptors in rat striatum (Waelbroeck et al. 1990). Conspicuously, the affinity estimates of the antagonists obtained in guinea-pig uterus and rat striatum were strikingly similar. In addition, the binding affinities of pirenzepine, AF-DX 116, methoctramine and 4-DAMP at M_4 receptors in NG108-15 cells (Michel et al. 1989) correlate well with their functional potencies in the guinea-pig uterus (this study).

Biochemical studies with NG108-15 cells (Harden et al. 1986) and cloned muscarinic receptors (Peralta et al. 1988; Ashkenazi et al. 1989) revealed that m4 receptors strongly inhibit adenylate cyclase. Moreover, m4 receptors have also been shown to mediate stimulation of phosphoinositide hydrolysis (Peralta et al. 1988; Ashkenazi et al. 1989). Both second messenger responses have been reported to occur upon muscarinic stimulation of the immature guinea-pig uterus (Leiber et al. 1984; Marc et al. 1986).

We therefore hypothesize that the isolated guinea-pig uterus preparation may serve as a simple functional assay system to study the pharmacology of M_4 receptors. To lend further support to this notion, molecular biological studies are underway to verify that m4 mRNA is in fact expressed in this tissue.

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