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Characterization of the muscarinic receptor subtype(s) mediating contraction of the guinea-pig lung strip and inhibition of acetylcholine release in the guinea-pig trachea with the selective muscarinic receptor antagonist tripitramine

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1 The muscarinic receptor subtypes mediating contraction of the guinea-pig lung strip and inhibition of the release of acetylcholine from cholinergic vagus nerve endings in the guinea-pig trachea *in vitro* have previously been characterized as M_2-like, i.e. having antagonist affinity profiles that are qualitatively similar but quantitatively dissimilar compared to cardiac M_2 receptors. The present study sought to establish definitely the identity of these receptor subtypes by using the selective muscarinic receptor antagonist, tripitramine. Guinea-pig atria and guinea-pig trachea (postjunctional contractile response) were included for reference.

2 It was found that tripitramine antagonized methacholine-induced contractions of the guinea-pig lung strip with a pK_B value of 8.76 ± 0.05. Both the parallel shifts of the concentration-response curves and the slope of the Schild plot being not significantly different from unity (when antagonist preincubation was for 2 h) indicated the involvement of a single population of receptors in the contractile response. From the pK_B values obtained with tripitramine and a range of other selective muscarinic receptor antagonists (cf. Roffel *et al*., 1993), this single population of receptors can only be classified as M_2-like.

3 Tripitramine antagonized methacholine-induced negative chronotropic and inotropic responses in guinea-pig right and left atria with apparent pK_B values of 9.4–9.6. However, such values were only obtained when antagonist preincubation was relatively long and/or antagonist concentration relatively high (e.g. with 1 h at 100 or 300 nM but 3 h at 30 nM). It thus appears that low concentrations of tripitramine do not readily equilibrate with M_2 receptors in guinea-pig atria nor with M_2-like receptors in the guinea-pig lung strip.

4 Tripitramine increased electrical field stimulation-induced cholinergic twitch contractions in guinea-pig trachea in concentrations of 0.3–100 nM, by blocking prejunctional muscarinic inhibitory autoreceptors; with higher concentrations, twitch contractions were progressively diminished, as a result of blocking postjunctional M_3 receptors (apparent pK_B value 6.07 ± 0.15). The pEC_{20} value ($-\log$ concentration that increases twitch by 20% of maximum) was 8.29 ± 0.08, which would suggest that M_4 receptors are involved in this response.

5 Oxotremorine-induced inhibition of the release of prelabelled [^{3}H]-acetylcholine from guinea-pig trachea, under conditions where there is no auto-feedback, was blocked by tripitramine (2 h preincubation) with a pK_B value of 8.56 ± 0.06. The slope of the corresponding Schild plot was not significantly different from unity, which together with the parallel shifts of the concentration-response curves indicated the involvement of a single muscarinic receptor subtype.

6 Since the pK_B value for tripitramine at prejunctional receptors in guinea-pig trachea is in between the affinities towards M_2 and M_4 receptors, correlation plots were constructed to compare the pK_B values obtained with tripitramine and a range of other selective muscarinic receptor antagonists (cf. Kilbinger *et al*., 1995) to reported affinities at M_1–M_4 receptors. This showed rather similar distribution patterns of the data points around the line of equality in the case of M_2 and M_4 receptor subtypes. However, the correlation coefficient was markedly better for M_2 (0.9667) than for M_4 (0.5976). Since recent evidence suggests that M_4 receptors are not expressed in cholinergic nerves from guinea-pig trachea, it is concluded that prejunctional muscarinic autoinhibitory receptors in this tissue exhibit an atypical M_2 type character, with a pharmacological profile distinct from cardiac M_2 receptors.

Keywords: Muscarinic receptor, subtypes of; prejunctional muscarinic autoreceptor; lung strip, guinea-pig; trachea, guinea-pig; atria, guinea-pig

Introduction

Muscarinic receptor subtypes have been shown to be involved in the control of airway smooth muscle tone and airway diameter. Thus, M_1 receptors are localized to sympathetic nerves (Maclagan *et al*., 1989) or parasympathetic ganglia (see Ten Berge *et al*., 1995 and references cited therein) where they facilitate neurotransmission. M_2(-like) receptors are found prejunctionally on parasympathetic cholinergic as well as on sympathetic adrenergic nerve endings (for references see below), where they serve to control neurotransmitter release. M_2 as well as M_3 receptors are found in smooth muscle (Roffel *et al*., 1988; Haddad *et al*., 1991). Among these smooth muscle muscarinic receptor subtypes, M_3 receptors are most directly involved in mediating contraction, as has been elaborately shown in guinea-pig isolated central airway smooth muscle preparations (trachea, main bronchi) as well as in peripheral

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human bronchi *in vitro* (Mutschler *et al*., 1988; Roffel *et al*., 1990; 1993; Kilbinger *et al*., 1991; Haddad *et al*., 1991; Watson *et al*., 1995), and in rat isolated whole lung *in vitro* (Post *et al*., 1991) and mouse and guinea-pig lung *in vivo* (Howell *et al*., 1991; Garssen *et al*., 1993). By contrast, we have previously shown that the muscarinic receptor subtype involved in contraction of the guinea-pig lung strip preparation *in vitro* is not homogeneously M_3, but more closely resembles the cardiac M_2 type or represents a mixture of M_2 with M_3 or M_4 receptors (Roffel *et al*., 1993).

The muscarinic receptor subtype mediating inhibition of acetylcholine release from parasympathetic cholinergic vagus nerve endings in guinea-pig airways has been mainly characterized as M_2, based on the fact that M_2 selective muscarinic antagonists such as gallamine and methoctramine increase nerve or field stimulation-induced constriction *in vivo* (Fryer & Maclagan, 1984; 1987; Del Monte *et al*., 1990; Watson *et al*., 1992) and *in vitro* (Yang & Biggs, 1991; Watson *et al*., 1992; Ten Berge *et al*., 1993). However, by measuring the release of pre-labelled [^{3}H]-acetylcholine under conditions where there is no auto-feedback, and applying Schild analysis, we have found that muscarinic antagonists display affinities towards these autoreceptors that indicate the involvement of M_4 rather than M_2 receptors (Kilbinger *et al*., 1995). The inability of most M_2 selective antagonists to discriminate clearly between M_2 and M_4 receptor subtypes (cf. Doods *et al*., 1993) may in fact suggest that these previous findings provide evidence for the involvement of the M_4 receptor subtype. In support of our findings, Patel and colleagues (1995) have shown that pirenzepine is surprisingly potent at muscarinic autoinhibitory receptors in the guinea-pig trachea.

With the advent of the new selective muscarinic receptor antagonist tripitramine, which discriminates at least 40 fold between M_2 and M_4 (or M_1) receptors (and more than 1000 fold between M_2 and M_3) (Melchiorre *et al*., 1993), it appeared that the primary drawback in the above investigations, i.e. the lack of muscarinic antagonists displaying high selectivity between various muscarinic receptor subtypes and especially M_2 and M_4, could be circumvented. We therefore established the affinities of tripitramine towards postjunctional muscarinic receptors in guinea-pig lung strip and prejunctional receptors in guinea-pig trachea, in order to identify definitely the muscarinic receptor subtype(s) involved in these cholinergic responses. The actions of tripitramine in guinea-pig atria (M_2 receptor subtype) and trachea (M_3 receptor subtype) were taken into account to serve as a reference, and also because of recent indications of non-equilibrium complications with tripitramine (Melchiorre *et al*., 1995; Chiarini *et al*., 1995).

Methods

Guinea-pig trachea and lung strip contraction experiments

Guinea-pig tracheal smooth muscle and lung strip preparations were set up for contraction experiments as described previously (Roffel *et al*., 1993). Briefly, adult (600–900 g) guinea-pigs of either sex (Charles River, Kisslegg, Germany) were terminated and the tracheae and lungs rapidly removed. The tracheae were carefully prepared free of mucosa, connective tissue and epithelium in Krebs-Henseleit (KH) buffer solution (composition in mM: NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and D-glucose 5.55; gassed with 95% O_2 and 5% CO_2; pH 7.4) at 37°C. Strips of peripheral lung tissue ($15 \times 2 \times 2$ mm) were cut from the body of a lower lobe with the longitudinal axis of the strip parallel to the bronchus, or from the peripheral margin of the lope. Lung strips and single-ring tracheal preparations were mounted for isotonic recording (0.3 g preload) at 37°C. After equilibration, preparations were precontracted twice with methacholine (0.1, 1, 10 and 0.1, 1, 10 and 100 μM) before construction of the control methacholine cumulative concentration-response curve. After tripitramine preincubation (1–300 nM for lung strip, 0.3–3 μM for trachea) for the time periods indicated (30 min, 1 or 2 h) the methacholine concentration-response curve was repeated. Antagonist-induced rightward shifts of the concentration-response curves were corrected for spontaneous shifts as determined in two out of eight strips in each experiment. Schild plots were constructed in four (2 h incubation) or five (1 h incubation) separate experiments in the case of guinea-pig lung strip. pK_B values were calculated for each concentration of antagonist tested and the means (±s.e.mean) presented (Mackay, 1978).

Electrical field stimulation-induced contractions of guinea-pig trachea

Electrical field stimulation (EFS)-induced contractions of guinea-pig tracheal single-ring preparations were performed based on the method described by Ten Berge *et al*. (1993). Briefly, the preparations were equilibrated as above and precontracted twice with methacholine (1 and 10 and 0.1–300 μM, respectively). After washout of the latter methacholine contraction, EFS (100 mA, 0.8 ms, 31 Hz, for 4 s every 80 s, delivered via two platinum electrodes on opposite sides of the preparation, 0.7 cm apart) was applied by a constant current biphasic square wave stimulator. After EFS-induced cholinergic twitch contractions had become constant (at least 30 min), tripitramine was added in a cumulative fashion (0.1 nM–3 μM), allowing each concentration to reach apparent equilibrium (15–20 min). The effect of tripitramine on EFS-induced twitch contractions was expressed as a percentage of the twitch height just before the first addition of the drug; pEC_{20}- (the negative logarithm of the concentration yielding 20% of the maximal increase in twitch height) and pIC_{50}- (the negative logarithm of the concentration inhibiting the maximal twitch height by 50%) values were calculated.

Tripitramine was studied in duplicate in 8 (absence of epithelium) or 9 (presence of epithelium) experiments.

Negative chronotropic and inotropic responses in guinea-pig atria

Guinea-pig atria were set up for measurement of negative inotropic and chronotropic responses according to standard procedures (Roffel *et al*., 1994). Briefly, after the animal had been killed, the heart was rapidly removed and cleaned in KH buffer solution at room temperature. Atria were mounted for isometric recording at a resting tension of 0.5 g in 20 ml organ baths (KH, 32°C), by means of Grass FT.03 force-displacement transducers connected to Grass 7P1 amplifiers in a Grass 79D Polygraph. The left atrial preparations were electrically stimulated at a rate of 3 Hz with pulses of 3 ms duration, with a constant current biphasic square wave stimulator. Stimulus current was 1.5 fold the threshold with a minimum of 7.5 mA, in order to assure constant maximal contractility during the experiment. The atria were equilibrated for at least 30 min with at least three washings, until contractility and heart rate, respectively, became constant. Methacholine cumulative concentration-response curves in the absence and presence of tripitramine (1–300 nM) were performed with antagonist preincubation periods as indicated (1, 2 or 3 h). One or two concentrations of antagonist were tested on a single preparation, the subsequent concentration being ten (occasionally three) times higher than the previous one; each concentration was tested in at least 3 different experiments. Tissue responses were measured as changes in isometric tension of the electrically paced left atria, and in heart rate of the spontaneously beating right atria, and were calculated as a percentage of the initial tension or rate at the start of each individual concentration-response curve.

Schild plots were constructed in three (right atria) or four (left atria, 1 h incubation) separate experiments including at least three antagonist concentrations. pK_B values were calculated for each concentration of antagonist tested and the means (±s.e.mean) presented (Mackay, 1978).

Release of [^{3}H]-acetylcholine from the guinea-pig trachea

Release of [^{3}H]-acetylcholine from epithelium-free trachea strips was studied as described previously (Kilbinger *et al.*, 1991). Briefly, the strips were suspended under a tension of 2 g in a 2 ml organ bath in a physiological salt solution (composition in mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, D-glucose 5.6 and choline chloride 0.001) at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The preparations were incubated for 1 h with [^{3}H]-choline (2.5 μCi ml^{-1}) during which the tissue was stimulated electrically with square wave monophasic pulses of 20 Hz and 1 ms duration, for 5 s every 30 s (Grass S6 stimulator), via two platinum electrodes that were positioned parallel to the strips (distance 0.6 cm; voltage drop 10 V cm^{-1}). The strips were then superfused (2 ml min^{-1}) with the physiological salt solution which contained in addition 10 μM hemicholinium-3. After washing out unincorporated [^{3}H]-choline for 130 min (experiments with 30 nM tripitramine) or 90 min (experiments with >30 nM tripitramine) the superfusate was collected in 3 min fractions and the tritium content of the samples measured by liquid scintillation spectrometry. The strips were stimulated electrically at 9 min (S1), 51 min (S2), 93 min (S3), 135 min (S4) and 165 min (S5) after the end of the washout period. Each stimulation period consisted of 300 impulses applied in trains of 10 pulses at a frequency of 100 Hz every 10 s. The stimulation-evoked outflow of ^{3}H radioactivity was calculated from the difference between the total outflow during and after stimulation, and the basal outflow calculated by interpolation from two samples before and after stimulation. Previous experiments have shown that the electrically-evoked outflow of ^{3}H radioactivity from this preparation consists only of [^{3}H]-acetylcholine (Kilbinger *et al.*, 1991). Oxotremorine was added to the medium in cumulatively increasing concentrations 30 min before S2, S3 and S4 and 18 min before S5 (see Figure 4). Tripitramine was present in the superfusate from the beginning of the washout period onwards. Accordingly, 30 nM tripitramine was present in the medium from 154 min before the lowest concentration of oxotremorine was added. Concentrations of 0.1, 0.3 and 1 μM tripitramine were added 114 min before the first concentration of oxotremorine. Concentration-response curves were constructed by expressing the ratio Sx/S1 in the presence of oxotremorine as a percentage of the equivalent ratio obtained in control experiments without oxotremorine. The concentrations which produced half-maximal inhibition of [^{3}H]-acetylcholine release (EC_{50}) were determined graphically from individual concentration-response curves on a semilogarithmic plot. pA_2-values were determined from Schild plots by regression analysis; pK_B values were calculated as described above.

Drugs

[Methyl-^{3}H]-choline chloride (NEN, Dreieich, Germany), methacholine chloride (Aldrich, Milwaukee, WI, USA), (−)-isoprenaline hydrochloride, oxotremorine sesquifumarate, choline chloride, hemicholinium-3 bromide (Sigma, St. Louis, MO, U.S.A.), tripitramine (gift from Dr C. Melchiorre, University of Bologna, Italy).

Data analysis

Statistical significance of differences was estimated by use of Student's *t* test and significance was assumed at the 5% level.

Results

Tripitramine antagonism of contractions of the guinea-pig lung strip

Methacholine-induced contractions of the guinea-pig lung strip ($-\log EC_{50} = 5.94 \pm 0.03$, 9 experiments) were clearly blocked by tripitramine concentrations of 10 nM and higher, the control concentration response curves shifting to the right in a parallel fashion (Figure 1a). Rightward shifts were directly proportional to antagonist concentrations with 2 h antagonist preincubation (Schild slope 1.10 ± 0.11, not significantly different from unity), but not with 1 h (Schild slope 1.45 ± 0.08, significantly different from unity, $P<0.01$) (Figure 1b). As a consequence, apparent pK_B values as calculated per concentration of tripitramine tested were similar in the case of 2 h incubation (yielding an average value of 8.76 ± 0.05, $n=16$), but concentration-dependent (and significantly different between 10–30 and 100–300 nM, $P<0.001$) in the case of 1 h (Table 1). There was no detectable or reliably measurable shift of the methacholine concentration-response curves with 1 nM tripitramine at either preincubation time. With 3 nM tripitramine there was a small shift at 2 h incubation, yielding an apparent pK_B value that was significantly ($P<0.02$) lower than at the higher concentrations. Depression of the maximum contraction was not observed.

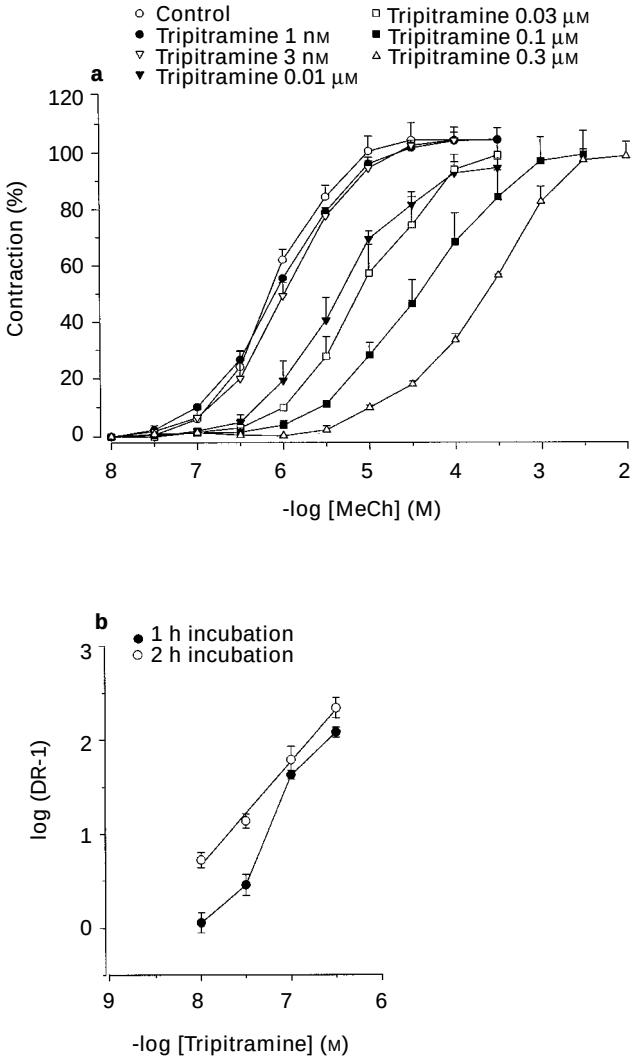

Figure 1 (a) Cumulative concentration-response curves for methacholine (MeCh)-induced contractions (expressed as a percentage of the maximum to methacholine in the second precontraction, see Methods) in the guinea-pig lung strip in the absence and presence of different concentrations of tripitramine. Tripitramine preincubation time was 2 h; data points represent means of 4 independent experiments; vertical lines show s.e.mean. (b) Schild plots for the antagonism of methacholine-induced contractions in the guinea-pig lung strip by tripitramine as shown in (a) (2 h antagonist incubation), and with 1 h antagonist incubation. Data points represent means and vertical lines s.e.mean of 4 (2 h) or 5 (1 h) independent experiments.

Table 1 Apparent pK_B values for tripitramine in the guinea-pig lung strip, as calculated per concentration of antagonist tested, with varying antagonist preincubation time

		Apparent pK_B (-log M)	
		1 h incubation	*2 h incubation*
Tripitramine	1 nM	ND	ND
	3 nM	ND	8.41±0.08*
	10 nM	8.05±0.10**	8.72±0.08
	30 nM	7.98±0.11**	8.66±0.07
	0.1 μM	8.63±0.05	8.79±0.14
	0.3 μM	8.60±0.05	8.86±0.11

Apparent pK_B values were calculated for each concentration of antagonist according to: $pK_B = -\log$ ([antagonist].(dose-ratio - 1)$^{-1}$), and are presented as means±s.e.mean of 5 (1 h preincubation) or 4 (2 h) independent experiments. ND – no detectable or reliably measurable shift of the methacholine concentration-response curves. *Significantly different from the average pK_B value obtained with 2 h incubation at the higher concentrations: $P<0.02$. **The average pK_B value at 10–30 nM was significantly different from that obtained with 1 h incubation at 0.1–0.3 μM: $P<0.001$.

Tripitramine antagonism of negative chronotropic and inotropic responses in guinea-pig atria

Methacholine-induced negative chronotropic responses in spontaneously beating guinea-pig right atria ($-\log EC_{50} = 5.89 \pm 0.05$; $n=12$) and inotropic responses in electrically paced left atria (6.60±0.08, $n=14$) were antagonized by tripitramine concentrations of 3 nM and higher in a parallel but not in a directly proportional fashion (1 h antagonist preincubation) (Figure 2a), resulting in Schild plots with slopes significantly different from unity (1.62±0.11 for right atria, $P<0.02$; 1.70±0.09 for left atria, $P<0.005$). An additional shift was observed with lower (30 nM) but not with higher (0.1 μM) concentrations of tripitramine with longer incubation periods (2 or 3 h) (Figure 2a, b). As a consequence, apparent pK_B values as calculated per concentration of tripitramine were concentration-dependent (and significantly different between 3–10 and 100–300 nM, $P<0.001$) with 1 h preincubation, but similar with 2 and 3 h incubation in the case of 30 and 100 nM (Table 2). There was no reliably measurable shift of the methacholine concentration-response curves with 1 nM tripitramine at either preincubation time; depression of the maximum responses was not observed.

Tripitramine antagonism of contractions of the guinea-pig trachea

Methacholine concentration-contractile response curves in guinea-pig trachea were shifted to the right by tripitramine (0.3–3 μM, 30 min antagonist preincubation) in a parallel fashion and without depression of the maximum, yielding an apparent pK_B value of 6.07±0.15 ($n=7$).

The effect of tripitramine on EFS-induced cholinergic twitch contractions in guinea-pig trachea

EFS-induced twitch contractions of guinea-pig tracheal ring preparations, either with or without epithelium, were increased by tripitramine concentrations of 0.3–100 nM (Figure 3). Maximum increases amounted to 180±13% ($n=9$) and 203±16% ($n=8$) of basal, respectively (not significantly different from each other); with higher concentrations of tripitramine twitch contractions were progressively blocked. pEC_{20}- and pIC_{50}- values were 8.21±0.06 and 5.69±0.07, respectively, in the presence, and 8.29±0.08 and 5.81±0.09, respectively, in the absence of epithelium (no significant difference with epithelium). The effect of 0.1–100 nM tripitramine was also established with 75–90 min contact time per concentration (2 or 3 cumulative concentrations per preparation), with virtually identical results (not shown).

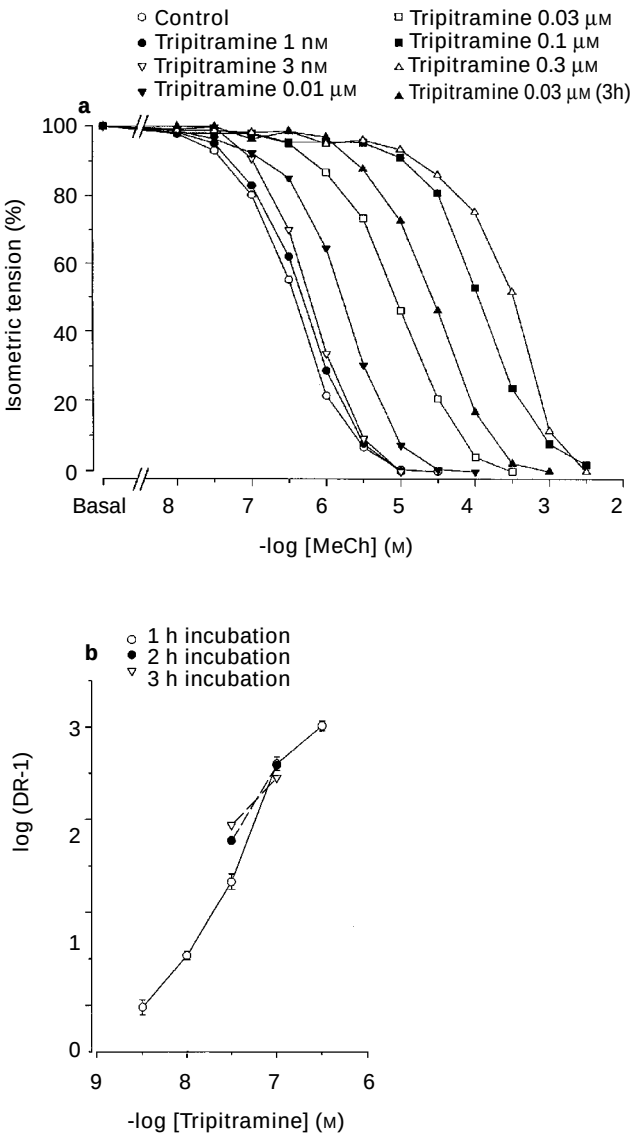

Figure 2 (a) Cumulative concentration-response curves for methacholine (MeCh)-induced inotropic responses (expressed as a percentage of the contractile force obtained just before each individual curve, see Methods) in electrically paced guinea-pig left atria in the absence and presence of different concentrations of tripitramine. Tripitramine preincubation time was 1 h, except 30 nM depicted as closed upward squares. Data points represent means of 5 or 6 independent experiments; errors bars (which amounted to 9% isometric tension or less) have been omitted for reasons of clarity. (b) Schild plot for the antagonism of methacholine-induced negative inotropic responses in guinea-pig left atria by tripitramine as shown in (a) (1 h antagonist incubation), and log (DR-1)-values obtained with 2 or 3 h antagonist incubation at 30 and 100 nM. Data points represent means and vertical lines s.e.mean of 5–6 independent experiments (1 h incubation), or means of 3–4 independent experiments (2 and 3 h incubation); in this latter case, error bars (which were of similar magnitude as with 1 h incubation) have been omitted for reasons of clarity.

Tripitramine antagonism of inhibition of acetylcholine release from guinea-pig trachea in the absence of autofeedback

In control experiments the release of [^{3}H]-acetylcholine evoked by S2, S3, S4 and S5 was 87±5, 82±8, 81±6 and 67±7% ($n=6$), respectively, of that caused by S1 (1131±

Table 2 Apparent pK_B values for tripitramine in guinea-pig right and left atria, as calculated per concentration of antagonist tested, with varying antagonist preincubation time

		Apparent pK_B (-log M)			
		Right atria	*Left atria*		
		1 h	*1 h*	*2 h*	*3 h*
Triptamine	1 nM	ND	ND	–	–
	3 nM	8.61±0.14	8.47±0.08	–	–
	10 nM	8.68±0.13	8.54±0.11	–	–
	30 nM	9.01±0.27	8.85±0.08	9.30±0.10	9.46±0.21
	0.1 μM	9.37±0.08	9.60±0.07	9.58±0.06	9.45±0.04
	0.3 μM	9.46±0.09	9.50±0.05	–	–

Apparent pK_B values were calculated for each concentration of antagonist according to: $pK_B = -\log([\text{antagonist}].(\text{dose-ratio} - 1)^{-1})$, and are presented as means±s.e.mean of 4–7 (right atria), 5–6 (left atria, 1 h incubation), or 3–4 (left atria, 2 and 3 h incubation) independent experiments. ND – no reliably measurable shift of the methacholine concentration-response curves.

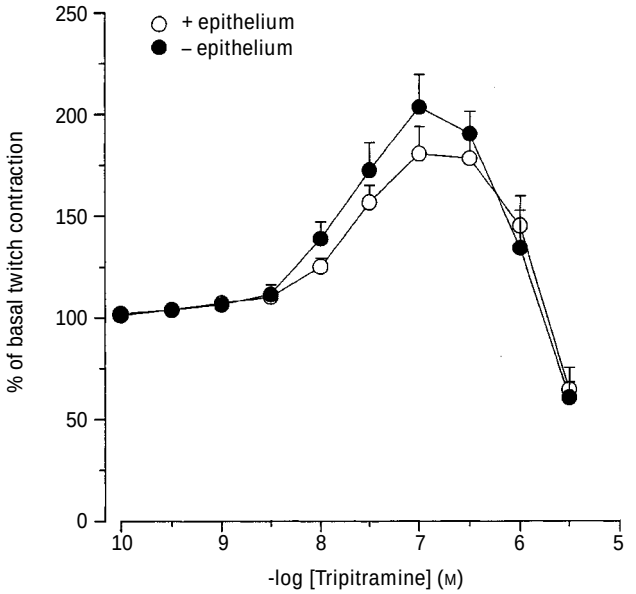

Figure 3 The effect of cumulative tripitramine administration on electrical field stimulation (100 mA, 0.8 ms, 31 Hz, for 4 s every 80 s)-induced cholinergic twitch contractions of guinea-pig tracheal ring preparations in the absence and presence of epithelium. Responses are expressed as a percentage of the basal twitch height (i.e. just before administration of the lowest tripitramine concentration) and represent means and vertical lines s.e.mean of 8 and 9 experiments, respectively, each performed in duplicate.

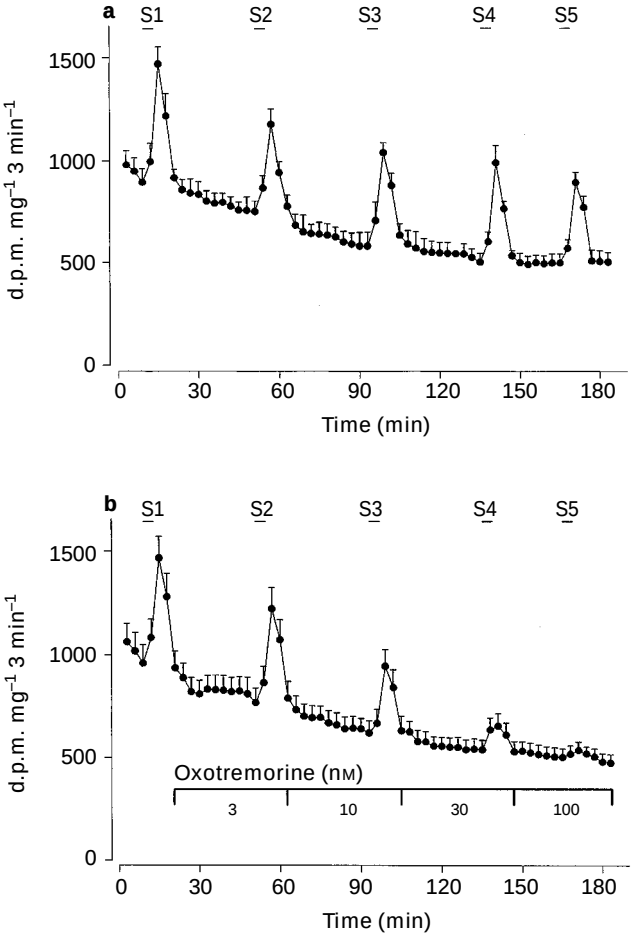

Figure 4 (a) The field stimulation-induced release of [^{3}H]-acetylcholine from guinea-pig tracheal strips that had been preincubated with [^{3}H]-choline. Stimulation periods are indicated at the top of the figure (S1–S5; 100 Hz, 300 pulses applied in trains of 10 pulses every 30 s). Time 0 min corresponds to the end of the washout period for unincorporated [^{3}H]-choline. Data points represent means and vertical lines s.e.mean of 6 independent experiments. (b) Inhibitory effect of oxotremorine on stimulation-induced release of [^{3}H]-acetylcholine. Horizontal bars indicate superfusion with increasing concentrations of oxotremorine ($n=8$).

228 d.p.m. mg^{-1}). Oxotremorine inhibited the evoked release in a concentration-dependent manner ($-\log EC_{50}$: 7.68±0.10, $n=8$) (Figures 4 and 5). The basal outflow of tritium was not changed by oxotremorine. Tripitramine behaved as a competitive antagonist and shifted the concentration-response curve for oxotremorine in a parallel manner to the right without affecting the maximal inhibitory effect (Figure 5a). Neither concentration of tripitramine affected the release of [^{3}H]-acetylcholine evoked by S1. This indicates that autoinhibition of acetylcholine release does not operate under the present stimulation conditions. The presynaptic pA_2 value for tripitramine was 8.34 (95% confidence limits: 8.07–8.73). The slope of the regression line in the Schild plot (Figure 5b) did not differ significantly from unity (1.15±0.11; $n=19$). When the slope was constrained to unity a pK_B value of 8.56±0.06 was calculated.

Discussion

Characterization of the muscarinic receptor subtype mediating contraction of the guinea-pig lung strip

Contraction of the guinea-pig lung strip preparation *in vitro* is not mediated by a homogeneous population of M_3 type receptors, in contrast to isolated airway smooth muscle preparations and to airway constriction in mouse, rat and guinea-pig lung (see Introduction), but rather by a (novel) subtype resembling most closely the cardiac M_2 receptor, or by a mixture of M_2 with M_3 or M_4 receptors (Roffel *et al*., 1993). This conclusion was based on the fact that by Schild analysis a number of subtype (M_3) selective muscarinic antagonists displayed affinities in guinea-pig lung that were very similar to

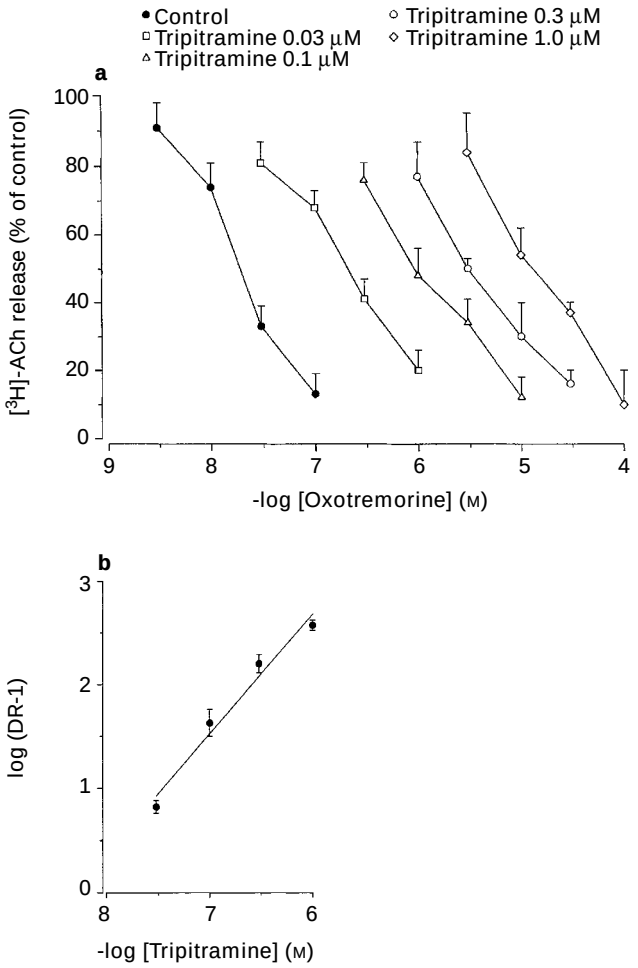

Figure 5 (a) Tripitramine antagonizes the inhibition by oxotremorine of the electrically-evoked release of [^{3}H]-acetylcholine from guinea-pig trachea. The effects of oxotremorine on the evoked release, in the absence and presence of increasing concentrations of tripitramine, are given as a percentage of the corresponding control values without oxotremorine. Data points represent means and vertical lines show s.e.mean, of 8, 5, 7, 3 and 4 determinations, respectively. (b) Schild plot for the antagonism between tripitramine and oxotremorine at the presynaptic muscarinic autoreceptor in guinea-pig trachea. Data were obtained from the dose-ratios between the individual concentration-response curves of oxotremorine in the presence of tripitramine and the average concentration-response curve of oxotremorine alone.

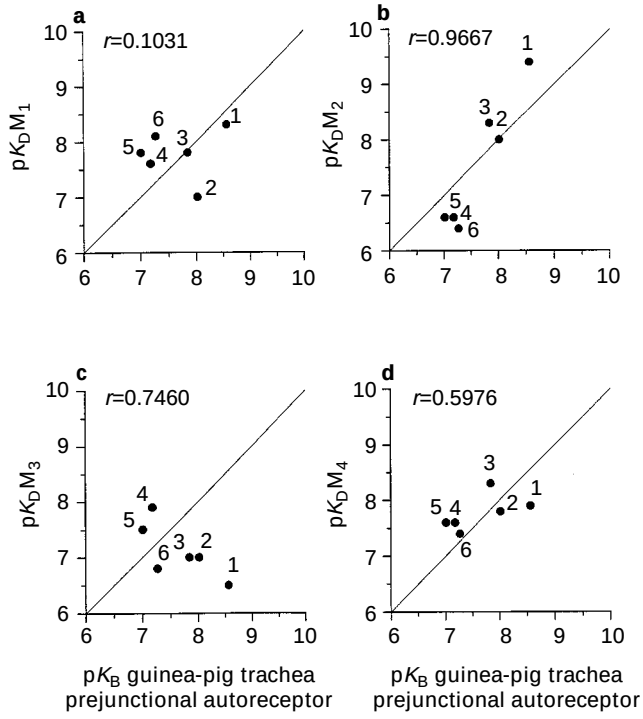

Figure 6 Correlation plots for the pK_B values obtained with receptor antagonists at prejunctional muscarinic autoinhibitory receptors in guinea-pig trachea and affinity estimates (pK_B or pK_i) for these compounds towards (a) M_1, (b) M_2, (c) M_3 and (d) M_4 muscarinic receptor subtypes, as compiled from the literature (Roffel *et al*., 1988; 1993; Buckley *et al*., 1989; Dörje *et al*., 1991; Doods *et al*., 1993; Lazareno & Birdsall, 1993; Maggio *et al*., 1994; Chiarini *et al*., 1995; Kilbinger *et al*., 1995; Lambrecht *et al*., 1995; Bräuner-Osborne & Brann, 1996). Note that the correlation coefficients given in the Figure regard the best fitting line through the data points, not the line of identity. The numbers correspond to: (1) tripitramine, (2) himbacine, (3) AQ-RA 741, (4) hexahydrosiladifenidol, (5) dicyclomine and (6) pirenzepine.

those observed at M_2 (but not M_4) receptors, whereas other (M_2 selective) compounds showed affinities in between those observed at M_2 and M_3 receptors, and consequently sometimes similar to M_4 (Roffel *et al*., 1993).

In the present study, the new muscarinic receptor antagonist tripitramine, which has been shown to discriminate relatively well between M_2 and M_4 and very clearly between M_2 and M_3 muscarinic receptor subtypes (Melchiorre *et al*., 1993; 1995; Lambrecht *et al*., 1995), was used in an attempt to identify unequivocally the receptor subtype(s) involved in this contractile response. From these experiments, the affinity (pK_B) obtained for tripitramine in antagonizing muscarinic agonist-induced contraction of the guinea-pig lung strip was concluded to be 8.76, as calculated from the shifts obtained with tripitramine 10–300 nM after 2 h of antagonist preincubation. Our results suggest that such an incubation period is sufficient to reach equilibrium with these concentrations since the slope of the Schild plot did not differ from unity under these conditions. By contrast, shorter antagonist preincubation (1 h) appeared inadequate to reach equilibrium since the lower concentration (10–30 nM) yielded lower apparent pK_B values than the higher concentrations (100–300 nM) under such conditions, resulting in a Schild slope of 1.45, and the higher concentrations also yielded slightly lower apparent pK_B values with 1 compared to 2 h incubation. Notably, tripitramine at 3 nM only produced reliably measurable shifts of the methacholine concentration-response curves with 2 h antagonist preincubation, but the apparent pK_B value calculated from these shifts was significantly lower than that obtained with higher concentrations of antagonist, indicating that for this low concentration 2 h preincubation is insufficient.

From the pK_B value of 8.76, the subtype of muscarinic receptor mediating contraction of the guinea-pig lung strip appears rather atypical. Thus, this pK_B value is lower than the affinities observed at M_2 receptors in guinea-pig and rat atria (9.37–9.60, this study; 9.14–9.85, Melchiorre *et al*, 1995; Chiarini *et al*., 1995) and at M_2 receptors in rabbit vas deferens and anococcygeus muscle (9.1, Lambrecht *et al*., 1995), but higher than those obtained at M_4 receptors (binding affinities obtained at native and cloned M_4 receptors being 7.93–8.19, Melchiorre *et al*., 1993; Maggio *et al*., 1994; functional affinity at M_4 receptors as found in rabbit anococcygeus muscle being 7.69, Lambrecht *et al*., 1995), and much higher than at M_3 receptors (affinities in guinea-pig trachea and guinea-pig and rat ileum being 6.1–6.8, this study and Chiarini *et al*., 1995). This pattern is reminiscent of that obtained with the M_2 selective muscarinic antagonists previously tested, i.e. gallamine, methoctramine, AF-DX 116 and AQ-RA 741 (Roffel *et al*., 1993), which, like tripitramine in the present study, exhibited slightly lower affinities at guinea-pig lung strip than at cardiac

M_2 muscarinic receptors. The present results with tripitramine do not support the possibility of a mixed population of receptor subtypes to any major extent, since the shape of the concentration-response curves was not significantly affected by tripitramine and the Schild plot (at 2 h antagonist incubation) had a slope of unity. As a result, we conclude that the muscarinic receptors mediating contraction in the guinea-pig lung strip consist of a single M_2-like population, with a pharmacological profile distinct from cardiac M_2 receptors. It should be added here that during a recent symposium on subtypes of muscarinic receptors (Levine & Birdsall, 1997), tripitramine was found to display affinities towards M_2 and M_2-like receptors in dog tracheal and chick ileum smooth muscle and in human detrusor muscle (prejunctional autoreceptors) that ranged from 8.34 to 8.85, suggesting that tripitramine has somewhat lower affinity towards non-cardiac M_2-(like) compared to cardiac M_2 receptors.

We have no explanation as to why Melchiorre and coworkers recently obtained a tripitramine affinity of 7.9 in guinea-pig lung strip *in vitro*, a value which is in between affinities towards M_2 and M_3 receptors as in the present study, but which in contrast to the affinity obtained in the present study agrees quite well with binding and functional affinities at M_4 receptors (see above). However, the putative involvement of M_4 receptors in contraction of the guinea-pig lung strip is not supported by the low affinities previously obtained with the M_3 selective antagonists (Roffel *et al*., 1993). Moreover, we have recently established the affinities in guinea-pig lung strip of two muscarinic receptor antagonists that display some selectivity for M_4 over M_2 receptors, i.e. trihexyphenidyl (affinity profile as compiled from Lazareno *et al*., 1990; Dörje *et al*., 1991; Waelbroeck *et al*., 1992; Bognar *et al*., 1992; Doods *et al*., 1993; Onali *et al*., 1994 being M_1 (9.0) = M_4 (8.8) > M_3 (8.3) > M_2 (7.8)) and tropicamide (M_4 (7.8) > M_1 (7.3) = M_2 (7.2) = M_3 (7.2); Lararenzo *et al*., 1990; Doods *et al*., 1993; Lazareno & Birdsall, 1993; Rinken, 1995). It was found that trihexyphenidyl behaved like the M_3 selective compounds, yielding a pK_B value (7.64 ± 0.10, $n=12$, estimated with concentrations ranging from 0.03–1.0 μM, slope of the Schild plot 1.15 ± 0.09, $n=3$) that resembled M_2 but not M_3 or M_4 affinities; by contrast, tropicamide yielded a pK_B value (6.57 ± 0.06, $n=14$, 0.3–30 μM, slope 1.03 ± 0.03) that was actually too low to correlate to any known muscarinic receptor subtype, behaviour which is reminiscent of trihexyphenidyl in rabbit iris sphincter and rabbit ear artery (Bognar *et al*., 1992; Darroch *et al*., 1992). Clearly, these compounds do not support a characterization of the muscarinic receptors mediating contraction of the guinea-pig lung strip as M_4.

Characterization of the actions of tripitramine in guinea-pig atria

The apparent affinity for tripitramine at cardiac M_2 receptors in guinea-pig right and left atria as found in the present study is 9.4–9.6, values which are well within the range of affinities observed in these tissues previously (9.20–9.85, Chiarini *et al*., 1995; Lambrecht *et al*., 1995; Melchiorre *et al*., 1995). These pK_B values of 9.4–9.6 were obtained after antagonist preincubation periods of 1 h and longer with tripitramine concentrations of 100 nM and above, and after 3 h of incubation with 30 nM tripitramine. Incubations of less than 3 h with 30 nM as well as for 1 h with lower concentrations (1–10 nM) apparently did not yield equilibrium, as judged from the lower apparent pK_B values obtained in those conditions, which also led to Schild slopes that were significantly higher than unity. The influence of incubation time on apparent pK_B values was especially obvious for 30 nM tripitramine in left atria, where increasing the antagonist incubation period from 1 to 2 and 3 h increased the apparent pK_B value by 0.45 and 0.61 log units, respectively. Based on these observations, it seems that the kinetic requirements for tripitramine at cardiac muscarinic M_2 receptors differ somewhat from those at guinea-pig lung strip muscarinic M_2-like receptors, where 2 h incubation is adequate to reach equilibrium with 10 and 30 nM tripitramine.

The reason for the lack of equilibrium with shorter incubation periods and low antagonist concentrations is unclear, but has been observed previously (Chiarini *et al*., 1995; Lambrecht *et al*., 1995; Melchiorre *et al*., 1995) and found to remain even after prolonging antagonist incubation to 3 h (Melchiorre *et al*., 1995). It is possible that the large molecular structure of tripitramine results in 'high affinity chromatography' when the compound diffuses into the tissue (K. Mohr, oral communication). This might be envisaged as the molecule being retained by interaction with various, non-muscarinic receptor, binding sites. Alternatively, a saturable removal process has been proposed as the mechanism underlying these observations (Chiarini *et al*., 1995). It should be noted that, at least at higher concentrations (approximately 1000 fold K_B), the actions of tripitramine at the M_2 receptor itself appeared to follow mass action laws, since there was normal additivity of antagonism with atropine (Chiarini *et al*., 1995).

Characterization of the prejunctional muscarinic receptor subtype mediating inhibition of acetylcholine release from guinea-pig trachea

Prejunctional muscarinic autoreceptors that control acetylcholine release from parasympathetic cholinergic vagus nerve endings in guinea-pig trachea have previously been characterized as either M_2 (Fryer & Maclagan, 1984; Watson *et al*., 1992; Ten Berge *et al*., 1993), M_2-like (Kilbinger *et al*., 1991) or M_4 (Kilbinger *et al*., 1995). As indicated in the Introduction, this uncertainty may well result from the lack of muscarinic receptor antagonists with adequate subtype selectivity, especially regarding the discrimination of M_2 and M_4 receptors.

In contrast to our expectations, the present study — employing tripitramine which exhibits unprecedented M_2/M_4 selectivity — has not unequivocally demonstrated whether M_2 and/or M_4 receptors are involved. Thus, in contraction experiments tripitramine clearly increased EFS-induced cholinergic twitch responses only at concentrations above 3 nM, i.e. concentrations suggestive of blocking M_4 receptors but higher than those required to block M_2 receptors. These observations were obtained both in the presence of epithelium, a condition which is similar to that employed in our previous study (Ten Berge *et al*., 1993), and in its absence, a condition which is similar to that employed when measuring the release of [^{3}H]-acetylcholine (see Kilbinger *et al*., 1991, 1995). In apparent agreement with the putative involvement of M_4 receptors that might be concluded from these observations, pEC_{20} values (cf. Ten Berge *et al*., 1993) were 8.2 to 8.3, i.e. closer to affinities obtained at M_4 (7.7–8.2, Melchiorre *et al*., 1993; Maggio *et al*., 1994; Lambrecht *et al*., 1995) than at M_2 receptors (9.1–9.8, Chiarini *et al*., 1995; Lambrecht *et al*., 1995; Melchiorre *et al*., 1995). The affinity of tripitramine towards prejunctional muscarinic autoreceptors in guinea-pig trachea, as derived from Schild analysis applied on the results of the [^{3}H]-acetylcholine release measurements (8.55), was similar to the observed pEC_{20} values, corroborating the correlation previously observed between such pEC_{20} values and (M_2) receptor affinities (Ten Berge *et al*., 1993), but at the same time was also slightly higher, and therefore in between affinities obtained for tripitramine towards M_2 and M_4 receptors (cf. above). A problem in the determination of presynaptic pK_B values is the presence of endogenous neurotransmitter in the synaptic cleft (Starke *et al*., 1989). As a result, the antagonist has to compete with both the endogenous and exogenous agonist (oxotremorine in the present study), and in this situation the presynaptic pK_B value may be underestimated (see Limberger *et al*., 1989 for an example). One procedure to avoid this difficulty is the use of very short bursts (< 100 ms) of pulses which cause a release of transmitter which is not subject to a negative feedback mechanism (Singer, 1988). Under this condition feedback inhibition of acetylcholine release from guinea-pig trachea does not develop: the antagonists scopolamine, AQ-RA 741

and hexahydrosiladifenidol which significantly increased the release caused by long trains (4.95 s) of 100 pulses applied at a frequency of 20 Hz (Kilbinger *et al*., 1991), did not enhance the release evoked by short trains (90 ms) of 10 pulses delivered at 100 Hz (Kilbinger *et al*., 1995). Similarly, neither concentration of tripitramine enhanced the release of acetylcholine in the present study. Hence, the presynaptic pK_B value for tripitramine was determined under conditions of no autoinhibition of acetylcholine release.

In an attempt to clarify further the matter of which muscarinic receptor subtype(s) inhibit(s) acetylcholine release from guinea-pig trachea, we prepared correlation plots in which the pK_B values obtained from Schild analysis of the release experiments were compared to affinity estimates towards M_1–M_4 receptors as presented in the literature (Figure 6). In this correlation analysis, those compounds that have been subjected to the Schild analysis were included, i.e. tripitramine (this study), himbacine, AQ-RA 741, hexahydrosiladifenidol, dicyclomine and pirenzepine (Kilbinger *et al*., 1995). Based on the correlation plots, it appears that prejunctional autoreceptors in guinea-pig trachea are not of the M_1 or M_3 type, but may equally well be of the M_2 or of the M_4 type, distribution of the data points around the line of equality being similar in these cases. The similar levels of (dis)agreement between pK_B values at prejunctional autoreceptors and M_2 and M_4 receptor affinity estimates may also be taken to indicate that both types of muscarinic receptor are jointly involved. This joint involvement may be especially suggested by the observations that himbacine and AQ-RA 741 (which are selective for M_2 over M_3 but hardly discriminate between M_2 and M_4) and the M_3 selective compounds (hexahydrosiladifenidol, dicyclomine, pirenzepine), which are up to 10 fold selective for M_4 over M_2, are not coincidentally distributed around the line of equality, and that increases in EFS-induced twitch contractions do indeed start at concentrations of tripitramine (0.3 nM) that will block M_2 but not M_4 receptors. However, Schild analysis of the release experiments yielded no indications for the involvement of more than one receptor subtype, with parallel rightward shifts of monophasic concentration-response curves and a Schild slope of unity being obtained. Moreover, the correlation coefficient was much better in the case of M_2 compared to M_4 receptors, i.e. 0.9667 ($P<0.001$) vs 0.5976 ($P>0.05$). Since recent observations show that guinea-pig tracheal parasympathetic nerves in culture only express M_2 receptor mRNA and protein — as measured by RT-PCR and immunoprecipitation, respectively (Fryer *et al*., 1996) - it is concluded that prejunctional muscarinic autoreceptors inhibiting acetylcholine release in guinea-pig trachea are of an atypical M_2 type, displaying a pharmacological profile as determined with well-known muscarinic receptor antagonists that is different from cardiac M_2 receptors. As already indicated above, this latter conclusion may have general validity, since affinity estimates for tripitramine in various non-cardiac tissues are clearly lower than in the heart.

In conclusion, the present study shows that the muscarinic receptor subtypes involved in contraction of the guinea-pig lung strip and in controlling the release of acetylcholine from guinea-pig tracheal parasympathetic vagus nerve terminals cannot be conclusively identified by use of an array of subtype selective muscarinic receptor antagonists in classical pharmacological experiments. Thus, whereas these receptors appear to be M_2-like in both tissues, the pK_B values were generally not identical to affinity estimates obtained towards cardiac M_2 receptors, and for some compounds more closely resembled those observed at M_4 receptors. The involvement of M_4 receptors in the parasympathetic nerves is precluded by the inability to detect M_4 receptor mRNA and protein (Fryer *et al*., 1996). However, similar experiments to establish the expression of muscarinic receptor subtype mRNAs and proteins (the latter by immunoprecipitation with subtype selective antibodies) in peripheral guinea-pig lung tissue, which might complement and substantiate the functional data presented here, have yet to be performed. The involvement of more than one receptor subtype in the responses investigated here is regarded unlikely, based on the parallel shifts in agonist concentration-response curves and the unity slope Schild plots that were obtained almost without exception (gallamine and methoctramine in guinea-pig lung strip - cf. Roffel *et al*., 1993). Finally, the use of tripitramine in the identification of muscarinic receptor subtypes, especially when aimed at discriminating M_2 and M_4 receptors, may prove somewhat less promising than originally anticipated, also because of the apparent lack of antagonism towards M_2 receptors at low concentrations.

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