Characterization of prejunctional muscarinic autoreceptors in the guinea-pig trachea

¹H. Kilbinger, R. Schneider, H. Siefken, D. Wolf & *G. D'Agostino

Pharmakologisches Institut, Universität Mainz, Obere Zahlbacher Str. 67, D-6500 Mainz, F.R.G. and *Istituto di Farmacologia, Università di Pavia, Viale Taramelli 14, I-27100 Pavia, Italy

- 1 The effects of ten muscarinic antagonists on electrically evoked [³H]-acetylcholine release and muscle contraction were compared in an epithelium-free preparation of the guinea-pig trachea that had been preincubated with [³H]-choline.
- 2 The M₃-selective antagonists UH-AH 37, 4-diphenyl-acetoxy-N-piperidine methobromide and *para*-fluorohexahydrosiladiphenidol were more potent in reducing the contractile response than in facilitating the evoked [³H]-acetylcholine release. Hexahydrosiladiphenidol did not discriminate between pre- and postjunctional effects. The rank order of the postjunctional potencies of the ten antagonists as well as the postjunctional pA₂ values obtained for hexahydrosiladiphenidol (7.95) and AQ-RA 741 (7.08) identified the muscular receptor as an M₃ subtype.
- 3 The M₂-selective antagonists methoctramine, AF-DX 116 and AQ-RA 741 were more potent in facilitating the evoked [³H]-acetylcholine release than in inhibiting the contractile response. The increase in release by low concentrations of methoctramine, AF-DX 116 and AQ-RA 741 was paralleled by an enhancement of the stimulation-evoked contractions.
- 4 Comparison of the pre- and postjunctional potencies of the M₁-, M₂- and M₃-selective antagonists suggests that autoinhibition of acetylcholine release is mediated via an 'M₂-like' receptor which differs from the cardiac type M₂ receptor in its relatively high affinity for hexahydrosiladiphenidol.

Keywords: Trachea; acetylcholine release; muscarinic autoreceptors; M₂ receptors; M₃ receptors

Introduction

The release of acetylcholine from guinea-pig airways can be inhibited by prejunctional muscarinic autoreceptors. This has been demonstrated in functional studies (Maclagan & Barnes, 1989; Starke *et al.*, 1989) and more recently in release experiments (D'Agostino *et al.*, 1990). Muscarinic receptors can be subdivided pharmacologically into at least three subtypes (M₁, M₂, M₃; for review see Hulme *et al.*, 1990). It has been suggested that the prejunctional autoreceptor in guinea-pig airways is an M₂ receptor since gallamine and methoctramine enhanced the electrically evoked contractions of the trachea *in vivo* (Fryer & Maclagan, 1984) and *in vitro* (Minette & Barnes, 1988; Watson *et al.*, 1989). To our knowledge, further studies to classify these autoreceptors with other antagonists are lacking.

The objective of the present study was to characterize in release experiments the muscarinic autoreceptor in the isolated trachea of the guinea-pig. For this purpose the pre- and postjunctional potencies of ten antagonists were compared on a novel preparation which allows the simultaneous determination of acetylcholine release and smooth muscle contraction (D'Agostino *et al.*, 1990). The antagonists tested are compiled in Table 1 and comprise those which are partially selective at M₁, M₂ or M₃ receptors. Complete concentration-response curves were performed for prejunctional (facilitation of electrically evoked acetylcholine release) and postjunctional (inhibition of evoked muscle contraction) effects, and the potencies of the antagonists were compared at the levels of the EC₅₀ values.

The smooth muscle receptor has been classified in the past as M₃ receptor according to its relatively high affinity for the antagonists 4-diphenyl-acetoxy-N-piperidine methobromide (4-DAMP), *para*-fluorohexahydrosiladiphenidol (pFHHSiD) and UH-AH 37, and its relatively low affinity to the antagonists pirenzepine, methoctramine, and AF-DX 116 (see Table 1). In the present study we have extended this classification by the determination of the postjunctional pA₂ values for the

Table 1 Postjunctional pA₂ values of antagonists used in this study as determined in the guinea-pig trachea

Antagonist	pA ₂	Reference
M ₁ -selective:		
Pirenzepine	6.9	Eglen & Whiting (1988)
M ₂ -selective:		
AQ-RA 741	7.0	This study
Methoctramine	6.1	Eglen & Whiting (1988)
AF-DX 116	6.3	Micheletti <i>et al.</i> (1987)
M ₃ -selective:		
4-DAMP	9.1	Eglen & Whiting (1988)
UH-AH 37	7.9	Eberlein <i>et al.</i> (1989)
HHSiD	8.0	This study
pFHHSiD	7.1	Eglen <i>et al.</i> (1990)
Non-selective:		
Ipratropium	9.6	Roffel (1990)

The table is based on studies in which pA₂ values were determined from Schild plots with slopes not different from unity. For abbreviations, see text.

M₃-selective antagonist hexahydrosiladiphenidol (HHSiD) (Mutschler & Lambrecht, 1984) and the M₂-selective antagonist AQ-RA 741 (Doods & Ziegler, 1989; Eberlein *et al.*, 1989).

A preliminary report of parts of this work has been given (Kilbinger *et al.*, 1990).

Methods

Tissue preparation

Guinea-pigs were killed by a blow to the head and exsanguinated. The trachea was rapidly removed, placed in a pre-warmed (37°C) and oxygenated (95% O₂ plus 5% CO₂) medium (composition in mmol l⁻¹: NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.05, NaHCO₃ 11.9, NaH₂PO₄ 0.42, D-glucose 5.6 and choline chloride 0.001) and gently cleaned from adhering

¹ Author for correspondence.

connective tissue. Unless stated otherwise, the epithelium was removed by rubbing the luminal surface with a moistened stem-pipe cleaner. This method was found consistently to cause complete removal of epithelium without damaging other tissue structures. A clip-connected strip was then prepared from one trachea as described in detail by D'Agostino *et al.* (1990).

Outflow of ^3H activity

The strip was suspended isometrically under a tension of 1 g in a 2 ml organ bath and superfused at a rate of 2 ml min^{-1} during a 30 min equilibration period. Superfusion was then stopped and the preparation incubated for 60 min with [^3H]-choline ($2.5\text{ }\mu\text{Ci ml}^{-1}$; final concentration $1\text{ }\mu\text{mol l}^{-1}$) under electrical stimulation. Square wave pulses of 1 ms duration were applied by two platinum electrodes positioned parallel to the trachea strip. The voltage drop between the electrodes was 10 V cm^{-1} and the stimulation frequency 20 Hz (trains of 100 impulses applied at intervals of 30 s). At the end of the incubation the strip was superfused with medium that contained in addition $10\text{ }\mu\text{mol l}^{-1}$ hemicholinium-3. After 90 min of washout the superfusate was collected continuously in 3 min fractions. In most experiments five stimulation periods were carried out on each preparation (S_1 – S_5 ; generally in 27 min intervals). Each stimulation period consisted of 600 impulses applied in trains of 100 impulses at a frequency of 20 Hz in intervals of 30 s. The first stimulation started 9 min after the end of the washout period. Tritium content of the samples was measured by liquid scintillation spectrometry and corrected for the counting efficiency by the external standardisation method. The stimulation-evoked outflow of ^3H activity was calculated from the difference between the total outflow during the stimulation plus the following 9 min, and the basal outflow. The basal outflow was assumed to decline linearly from the two samples immediately preceding the stimulation to the two samples 15–21 min after the onset of stimulation.

Testing of antagonists

Muscarinic antagonists were added after S_1 to the perfusion medium in cumulatively increasing concentrations so that complete concentration-response curves for both pre- and postjunctional effects were obtained in each experiment (cf. Figures 3 and 4). Depending on the concentrations tested, the antagonists were added to the medium either 24 min ($<10\text{ nmol l}^{-1}$), 18 min (0.01 – $1\text{ }\mu\text{mol l}^{-1}$) or 15 min ($>1\text{ }\mu\text{mol l}^{-1}$) before the onset of the respective stimulation. The prejunctional effects of the drugs were expressed as ratios S_x/S_1 (S_x : S_2 – S_5).

Contractions of the smooth muscle were recorded simultaneously with a force displacement transducer and displayed on a Hellige recorder. The peak tension developed by all twitches during S_1 were added up, and this total was compared to that obtained by adding up the individual responses in S_2 , S_3 , S_4 and S_5 .

Concentration-response curves for pre- and postjunctional effects were constructed by expressing the ratio S_x/S_1 in the presence of an antagonist as a percentage of the equivalent ratio obtained in control experiments in the absence of antagonists. The concentrations which produced half-maximal responses (EC_{50}) were determined graphically from individual concentration-response curves on a semilogarithmic plot.

Separation of labelled choline and acetylcholine

In some experiments the nature of radioactivity released by electrical stimulation was determined. In these studies the amount of [^3H]-choline used for labelling was increased to $5\text{ }\mu\text{Ci ml}^{-1}$, and superfusion rate was reduced to 1 ml min^{-1} . Neostigmine ($10\text{ }\mu\text{mol l}^{-1}$) and hyoscine (0.1 mol l^{-1}) were present in the superfusion medium from 30 min before the

stimulation onwards. [^{14}C]-choline (2 nCi) and [^{14}C]-acetylcholine (2–4 nCi) were added to each superfusate sample as internal standards. Labelled acetylcholine and choline were extracted from the medium by ion-pair extraction with tetraphenylborate/allylcyanoind and separated by thin-layer chromatography as described earlier (Kilbinger & Nafziger, 1985). The ion-pair extraction is selective for acetylcholine and choline, and phosphorylcholine in particular is not extracted from the aqueous into the organic phase (Barker *et al.*, 1972; Lindmar *et al.*, 1982). Recoveries were determined in separate experiments by adding authentic [methyl- ^3H]-choline and [acetyl- ^3H]-choline (each 10 nCi) to 5 ml of the physiological salt solution. The samples were carried through the above mentioned procedure of ion-pair extraction and thin layer chromatography and the recoveries were $97 \pm 3\%$ for acetylcholine and $88 \pm 5\%$ for choline (each $n = 3$).

Determination of postjunctional pA_2 values

The epithelium-free trachea was cut spirally, divided into sections 1–2 cm long and suspended isometrically under a tension of 1 g in a physiological salt solution (composition see above) at 37°C . Following an equilibrium period of 1 h the tissue was exposed, on 3 occasions, to $10\text{ }\mu\text{mol l}^{-1}$ of acetylcholine. Only when consistent responses were obtained to this concentration of acetylcholine were experiments performed. Cumulative concentration-response curves to oxotremorine were then constructed using successive concentrations incrementally spaced at 0.5 log intervals. After construction of the first concentration-response curve, the preparation was washed at 10 min intervals for 30 min, during which time the tension returned to baseline levels. The preparation was then exposed for 30 min to the antagonist, and a second curve to oxotremorine was constructed. All contraction heights were expressed as a percentage of the maximal effect of oxotremorine in the first curve. Parallel control experiments without antagonist were performed under identical conditions. These control experiments allowed for any correction to be made for changes in sensitivity to oxotremorine. The pA_2 values were determined from Schild plots by regression analysis (Arunlakshana & Schild, 1959).

Statistics

Results are given as means $\pm$ s.e.mean. EC_{50} values were converted to logarithmic form before the values from individual experiments were averaged and the s.e. were calculated. Statistical testing (two-tailed t test) was performed with the negative logarithms of the EC_{50} values. If one control group was compared with more than one group of treatments a one way analysis of variance was performed followed by Dunnett's test (Dunnett, 1964).

Drugs

[Methyl- ^3H]-choline (NEN, Dreieich, F.R.G.); AF-DX 116; AQ-RA 741 (11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one hydrochloride); pirenzepine dihydrochloride monohydrate; UH-AH 37 (6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)-acetyl]-11H-dibenzo-[b,e][1,4]diazepine-11-one hydrochloride) (all gifts from Thomae, Biberach, F.R.G.); 4-DAMP (4-diphenyl-acetoxy-N-piperidine methobromide; gift from Dr Barlow, Bristol, U.K.); hexahydrosiladiphenidol hydrochloride (HHSiD); *para*-fluorohexahydrosiladiphenidol hydrochloride (pFHHSiD) (gifts from Profs. Lambrecht and Mutschler, Frankfurt, F.R.G.); hyoscine hydrobromide; oxotremorine sesquifumarate (Sigma, St. Louis, MO, U.S.A.); ipratropium bromide (Boehringer, Ingelheim, F.R.G.); methoctramine hydrochloride (RBI, Natick, MA, U.S.A.).

Results

Outflow of ^3H activity from strips preincubated with $[\text{}^3\text{H}]$ -choline

In a first series of experiments the outflow of ^3H activity was characterized. Intermittent stimulation at 20 Hz caused an increase in outflow which was similar from epithelium-free strips (S_1 : 1284 ± 276 d.p.m. mg^{-1} ; $n = 5$) and from epithelium-containing preparations (S_1 : 1058 ± 253 d.p.m. mg^{-1} ; $n = 6$). Omission of calcium from the medium accelerated the basal outflow from both preparations (Figure 1). The stimulation-evoked outflow from epithelium-free strips was abolished, whereas the outflow from strips with the epithelium present was not reduced but slightly enhanced in the absence of extracellular calcium. Contractions of the trachealis muscle in response to electrical stimulation were abolished in both types of preparations.

The outflow of ^3H activity from epithelium-free strips was further analyzed after separation of $[\text{}^3\text{H}]$ -acetylcholine and $[\text{}^3\text{H}]$ -choline in the superfusate which contained neostigmine plus hyoscine. Electrical stimulation caused an increase in outflow of ^3H activity which consisted of $104 \pm 11\%$ ($n = 4$) of $[\text{}^3\text{H}]$ -acetylcholine (Figure 2). The outflow of $[\text{}^3\text{H}]$ -choline remained unchanged. The sum of labelled choline and acetylcholine in the samples of spontaneous outflow before and after stimulation accounted for $98 \pm 6\%$ (0–5 min) and $97 \pm 9\%$ (25–30 min), respectively, of the total ^3H radioactivity.

In some experiments the effects of oxotremorine on the evoked outflow of ^3H activity were studied. Strips were stimulated twice (S_1 , S_2 ; 20 Hz, 600 pulses) and $1 \mu\text{mol l}^{-1}$ oxotremorine was added 18 min before S_2 . In the presence of oxotremorine the electrically-evoked outflow of ^3H activity

Figure 1 The effect of calcium withdrawal on the outflow of ^3H activity from epithelium-free (a; $n = 5$) and epithelium-containing (b; $n = 6$) tracheal preparations preincubated with $[\text{}^3\text{H}]$ -choline ($5 \mu\text{Ci}$). Time '0 min' (abscissa scale) corresponds to the end of the 90 min washout period. Each stimulation period (S_1 , S_2 , S_3) consisted of 6 trains of 100 pulses delivered at intervals of 30 s at a frequency of 20 Hz. Calcium was omitted from the superfusion medium as indicated by the horizontal bar. Given are mean values; standard errors were 4–18% of corresponding means.

Figure 2 Outflow of total ^3H radioactivity (■), $[\text{}^3\text{H}]$ -acetylcholine (●) and $[\text{}^3\text{H}]$ -choline (▲) from epithelium-free tracheal strips that had been preincubated with $[\text{}^3\text{H}]$ -choline ($10 \mu\text{Ci}$). The tissue was stimulated electrically from 10–13 min (trains of 100 pulses delivered every 30 s at 20 Hz). Each point represents the mean of 4 experiments; vertical lines show s.e.mean.

from epithelium-free trachea strips was only $27 \pm 5\%$ ($n = 7$) of that elicited during S_1 . The calcium-independent outflow from strips with the epithelium present was not significantly affected by oxotremorine and was $96 \pm 17\%$ ($n = 4$) of that caused by S_1 .

Effects of muscarinic antagonists on outflow and contractions in epithelium-free strips

Strips were stimulated five times (S_1 – S_5) and the antagonists were added cumulatively. Figures 3 and 4 show representative time courses of the experiments. In the control experiments the outflow evoked by S_2 , S_3 , S_4 and S_5 was 81 ± 3 , 68 ± 3 ,

Figure 3 Effects of methoctramine on the field stimulation-evoked outflow of ^3H radioactivity from tracheal strips that had been preincubated with $[\text{}^3\text{H}]$ -choline. Stimulation periods are indicated at the top of the figure (S_1 – S_5 ; 20 Hz, 600 pulses applied in trains of 100 pulses every 30 s). Horizontal bar indicates superfusion with increasing concentrations of methoctramine ($n = 6$). Upper curve, control experiments ($n = 7$). Values are mean with s.e.mean indicated by the vertical lines.

Figure 4 Typical responses of the trachealis muscle to electrical field stimulation (S_1 – S_5) in the presence of increasing concentrations of AF-DX 116 and methoctramine. Upper panel, control experiment. Each stimulation period consisted of 6 trains of 100 pulses delivered at 20 Hz in 30 s intervals. Horizontal line, 1 g.

67 ± 3 and $56 \pm 3\%$ ($n = 19$), respectively, of that caused by S_1 . All antagonists produced concentration-dependent increases in the evoked outflow (Figure 5). Some antagonists showed bell-shaped prejunctional concentration-response curves which may be due to an unspecific inhibitory effect of the large concentrations on the evoked outflow. None of the compounds affected the basal outflow of tritium. The increase in outflow by low concentrations of the M_2 -selective antagonists methoctramine, AF-DX 116 and AQ-RA 741 was paralleled by a significant enhancement of the stimulation-evoked contractile response and only higher concentrations of these drugs inhibited the contractions. It should be noted that methoctramine up to $10 \mu\text{mol l}^{-1}$ failed to reduce significantly the postjunctional response. In separate experiments in which the effect of $50 \mu\text{mol l}^{-1}$ methoctramine was tested, a significant inhibition (by $71 \pm 7\%$, $n = 4$) of the electrically-evoked contraction was found. The M_3 -selective antagonists UK-AH 37, 4-DAMP and pFHHSiD displayed another pattern: stimulation-evoked contractions were already largely inhibited at concentrations that did not yet significantly increase the evoked outflow.

The negative logarithms of the pre- and postjunctional EC_{50} values are given in Table 2. M_2 -selective antagonists (methoctramine, AF-DX 116, AQ-RA 741) were more potent contraction was found. The M_3 -selective antagonists UK-AH 37, 4-DAMP and pFHHSiD at the postjunctional receptors. Surprisingly, HHSiD did not discriminate between pre- and

postjunctional muscarinic receptors. Likewise, hyoscine, ipratropium and pirenzepine had similar potencies at pre- and postjunctional receptors.

Since the relaxation response elicited by field stimulation (cf. Figure 4) might have been mediated by the release of nor-adrenaline, we have studied the effects of propranolol. The tracheal strips were stimulated twice (20 Hz, 600 pulses) and $1 \mu\text{mol l}^{-1}$ propranolol was added 18 min before the second stimulation. In the presence of propranolol the relaxation remained unchanged and the electrically evoked ^3H outflow was not affected ($111 \pm 6\%$ of the control value, $n = 4$). The pre- and postjunctional effects of the M_2 - and M_3 -selective compounds AF-DX 116 and 4-DAMP were also studied in the presence of $1 \mu\text{mol l}^{-1}$ propranolol. The $-\log$ of the pre- and postjunctional EC_{50} values for AF-DX 116 (7.58 ± 0.53 and 5.53 ± 0.06 ; $n = 3$) and for 4-DAMP (7.61 ± 0.12 and 8.85 ± 0.26 ; $n = 3$) were not different from the respective values in Table 2. All this shows that under the present experimental conditions a possible effect of endogenously released noradrenaline could not be detected, and that the relaxation of the smooth muscle is probably due to the release of a non-adrenergic non-cholinergic inhibitory transmitter.

Postjunctional pA_2 values

To our knowledge pA_2 values for the effects of HHSiD and AQ-RA 741 on guinea-pig trachealis smooth muscle have not

Table 2 Comparison of pre- and postjunctional potencies of muscarinic antagonists in the guinea-pig isolated trachea

Antagonist	$-\log EC_{50 \text{ pre}}$	$-\log EC_{50 \text{ post}}$	$\frac{EC_{50 \text{ pre}}}{EC_{50 \text{ post}}}$
Pirenzepine	7.08 ± 0.18	6.83 ± 0.10	0.56
Methoctramine	6.84 ± 0.16	$4.55 \pm 0.06^{**}$	0.005
AF-DX 116	7.48 ± 0.25	$5.55 \pm 0.05^{**}$	0.011
AQ-RA 741	7.53 ± 0.24	$6.24 \pm 0.09^{**}$	0.05
HHSiD	7.40 ± 0.10	7.26 ± 0.15	0.72
pFHHSiD	6.02 ± 0.16	$6.82 \pm 0.06^{**}$	6.3
UH-AH 37	6.70 ± 0.13	$7.58 \pm 0.03^{**}$	7.6
4-DAMP	7.78 ± 0.18	$8.89 \pm 0.11^*$	12.9
Hyoscine	8.85 ± 0.38	8.89 ± 0.21	1.1
Ipratropium	8.95 ± 0.23	8.53 ± 0.03	0.38

The $-\log EC_{50}$ values are mean $\pm$ s.e.mean of 4–8 experiments. Significance of difference between pre- and postjunctional $-\log EC_{50}$ values: * $P < 0.05$; ** $P < 0.01$ (paired t test). Values for methoctramine were compared by unpaired t test since prejunctional ($n = 6$) and postjunctional ($n = 4$) EC_{50} values were determined in separate experiments.

Figure 5 Effects of muscarinic receptor antagonists on outflow of ^3H activity (●) and smooth muscle contraction (■) evoked by electrical stimulation of epithelium-free tracheal preparations. Tracheal strips were stimulated 5 times and the antagonists added in cumulatively increasing concentrations before each stimulation period. Control experiments were run in parallel, and the effects of the antagonists are given as a percentage of the corresponding control value. Each point is the mean (s.e.mean shown by vertical lines) of 4–8 experiments. Significance of increases in ^3H outflow and muscle contractions: * $P < 0.05$; ** $P < 0.01$ (ANOVA followed by Dunnett's test).

been reported before. HHSiD (0.01, 0.1, 1.0 and $10 \mu\text{mol l}^{-1}$; each $n = 4$) and AQ-RA 741 (0.1, 0.3, 1.0 and $3.0 \mu\text{mol l}^{-1}$; each $n = 4$) caused parallel shifts to the right of the concentration-response curves for oxotremorine without affecting the maximal contraction. When the log (concentration ratio – 1) was plotted against log antagonist concentration, straight regression lines were obtained with slopes of 0.99 ± 0.08 (HHSiD; mean $\pm$ s.d.) and 0.97 ± 0.07 (AQ-RA 741). The pA_2 values (with confidence limits) were 7.95 (7.69–8.28) for HHSiD, and 7.08 (6.93–7.27) for AQ-RA 741.

Discussion

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

Intermittent field stimulation of the epithelium-free trachea strip that had been preincubated with [^3H]-choline causes an outflow of ^3H activity which for the following reasons is thought to be a reliable indicator for release of [^3H]-acetylcholine: the evoked outflow is calcium-dependent, and as shown recently (D'Agostino *et al.*, 1990), is abolished by tetrodotoxin. Furthermore, the electrically evoked outflow of ^3H activity consists of 100% [^3H]-acetylcholine, and the sum of labelled choline and acetylcholine during rest and field stimu-

lation is about 100% of the outflow of the total ^3H radioactivity (Figure 2). This suggests that a release of other [^3H]-choline metabolites does not contribute to the outflow of ^3H activity from the present epithelium-free preparation. On the other hand, most of the ^3H outflow evoked by field stimulation of an epithelium-containing trachea was [^3H]-phosphorylcholine in addition to [^3H]-acetylcholine, whereas field stimulation of an epithelium-free trachea preparation did not elicit a significant release of [^3H]-phosphorylcholine (Wessler *et al.*, 1990). The release of ^3H activity from the epithelium-containing trachea is not blocked by tetrodotoxin (Wessler *et al.*, 1990) and presumably arises from non-neuronal cells. In the present experiments the evoked outflow of this non-neuronal ^3H activity was neither reduced by the agonist oxotremorine nor by omission of calcium from the medium. On the other hand, oxotremorine strongly inhibited the evoked outflow of neuronal [^3H]-acetylcholine from the epithelium-free tracheal strip stimulated intermittently at 20 Hz.

Characterization of the prejunctional muscarinic receptor

Comparison of pre- and postjunctional potencies of the antagonists shows that the muscarinic receptors located at both sites of the cholinergic neuroeffector junction are pharmacologically different. The postjunctional receptor has been classified in the past as an M_3 receptor, and this has been corroborated in the present study (Table 1). Our pA_2 value obtained with the M_3 -selective antagonist HHSiD (7.95) agrees with the value found at the M_3 receptor of the ileal smooth muscle (8.22; Fuder *et al.*, 1985). Further, the present value for the cardioselective antagonist AQ-RA 741 (7.08) is close to the pA_2 value obtained at ileal M_3 receptors (6.55) and distinctly different from that at cardiac M_2 receptors (8.49; Doods & Ziegler, 1989). Finally, the rank order of the postjunctional $-\log \text{EC}_{50}$ values (Table 2) is similar to the rank order of the pA_2 values for these antagonists at the trachealis muscle (Table 1) and also identifies the muscular receptor as an M_3 receptor.

Pirenzepine did not discriminate between pre- and postjunctional effects, which suggests that the autoreceptor is not an M_1 receptor. Several lines of evidence indicate that the prejunctional receptor is similar to the M_2 subtype. Firstly, low concentrations of the M_2 -selective antagonists methoctramine, AF-DX 116 and AQ-RA 741 increased the contractile response to electrical stimulation, presumably due to selective facilitation of acetylcholine release. A similar enhancement of cholinergic neurotransmission through selective blockade of prejunctional muscarinic receptors by methoctramine has also been observed on the sphincter muscle of guinea-pig iris (Bognar *et al.*, 1990). Secondly, AQ-RA 741, AF-DX 116 and methoctramine were 20–200 times more potent at pre- than at postjunctional receptors. It should be noted that for methodological reasons the postjunctional $-\log \text{EC}_{50}$ value for methoctramine is somewhat too low (and hence the ratio $\text{EC}_{50} \text{ pre}/\text{EC}_{50} \text{ post}$ too low) since a possible inhibitory effect of low concentrations of methoctramine on muscle contraction is masked by the enhanced acetylcholine release. Nevertheless, the differences in pre- and postjunctional potencies are close to the differences between the pA_2 values at M_2 receptors in guinea-pig atria (methoctramine: 7.7–8.1, Melchiorre, 1988; AF-DX 116: 7.3, Micheletti *et al.*, 1987; AQ-RA 741: 8.5, Doods & Ziegler, 1989) and postjunctional M_3 receptors of the trachea (cf. Table 1). Thirdly, the M_3 -selective antagonists pFHHSiD, UH-AH 37 and 4-DAMP were 6–13 times more potent at the postjunctional than at the prejunctional receptor. Again, these differences in potencies correspond to the differences in pA_2 values determined on M_3 receptors (Table 1) and on cardiac M_2 receptors (4-DAMP: 7.6, Barlow *et al.*, 1976; UH-AH 37: 7.1, Doods & Ziegler, 1989; pFHHSiD: 6.0, Lambrecht *et al.*, 1988).

The pre- and postjunctional potencies of the non-selective antagonists hyoscine and ipratropium were similar. Ipratropium was included since this antagonist increased *in vivo* the

vagally induced bronchoconstriction even at very low doses which had no effect on postjunctional muscarinic receptors (Fryer & MacLagan, 1987). In our experiments ipratropium was the most potent of the ten antagonists at the autoreceptor, but ipratropium displayed no preferential effect at the prejunctional receptor. This was to be expected because the affinity of ipratropium for M_2 receptors (pA_2 8.8; Van Charldorp *et al.*, 1989) is not higher than that for M_3 receptors (pA_2 9.6; Roffel, 1990).

So far, the data of our study suggest that the prejunctional muscarinic receptor belongs to the M_2 subtype. However, HHSiD did not discriminate between pre- and postjunctional effects, although HHSiD is known to be about 50 times more potent on M_3 than on M_2 receptors (Mutschler & Lambrecht, 1984; Fuder *et al.*, 1985). Moreover, the difference in potency between hyoscine and HHSiD at the prejunctional muscarinic receptor (28 fold, Table 2) is much smaller than the difference in the affinities at cardiac M_2 receptors (630 fold) where pA_2 values of 9.1 (hyoscine, Fuder *et al.*, 1981) and 6.3 (HHSiD, Fuder *et al.*, 1985) were determined. The findings with HHSiD cannot be explained by the occurrence of a mixture of M_2 and M_3 receptors since the other selective antagonists clearly discriminated between both subtypes. Differences in the diffusion of HHSiD to pre- and postjunctional muscarinic receptors can also not account for the atypical behaviour of HHSiD because pre- and postjunctional effects were measured in the same experiment. The classification of muscarinic receptor subtypes

has recently been extended by the cloning of 5 distinct subtypes ($m1$ - $m5$; Bonner, 1989). The $m4$ gene encodes a muscarinic receptor with the greatest homology to the cardiac $m2$ receptor. Binding studies showed that the $m4$ gene product displayed a relatively high affinity not only for the M_2 -selective antagonists AF-DX 116 and methoctramine but also for HHSiD (Buckley *et al.*, 1989). However, classification of the autoreceptor in the guinea-pig trachea as an M_4 subtype appears premature until selective antagonists for the M_4 receptor become available.

In conclusion, the depolarization-evoked release of acetylcholine from the guinea-pig trachea is inhibited by an 'M₂-like' autoreceptor. The autoreceptor differs from the cardiac M_2 receptor in its relatively high affinity for HHSiD. The selective blockade of the prejunctional receptor by methoctramine, AF-DX 116 and AQ-RA 741 has postjunctional consequences and leads to enhanced muscle contraction. If the vagus nerve in human airways were also endowed with similar autoreceptors, such drugs could enhance vagally induced bronchoconstriction.

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