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Muscarinic cholinoceptor subtypes mediating tracheal smooth muscle contraction and inositol phosphate generation in guinea pig and rat

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Abstract

The effects of the muscarinic cholinoceptor antagonists atropine (non-selective), pirenzepine (M_1-selective), methoctramine (M_2-selective) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; M_3-selective) were examined on the responsiveness of guinea pig and rat tracheal tissue to acetylcholine and carbachol. Results indicate that smooth muscle contraction in isolated tracheal tissue from both species was mediated primarily by muscarinic M_3 cholinoceptors. The effects of atropine, pirenzepine and 4-DAMP were similar against the contractile actions of acetylcholine and carbachol in both species and in epithelium-intact and epithelium-denuded tissue. In contrast, differences in the effects of methoctramine in antagonising contractile responses to acetylcholine and carbachol were observed between the two species and following epithelium removal in the guinea pig. Thus, whilst this study has found that tracheal smooth muscle contraction in the guinea pig and rat is mediated primarily by muscarinic M_3 cholinoceptors, anomalies in the functional inositol phosphate generation results obtained with the muscarinic cholinoceptor antagonists highlight species differences in the actions of acetylcholine and carbachol in eliciting smooth muscle contraction suggesting the possible existence of functional non-M_3 muscarinic cholinoceptors.

Keywords: Muscarinic cholinoceptor; Airway; Inositol phosphate; Methoctramine; Atropine; Pirenzepine; 4-DAMP (4-Diphenylacetoxy-*N*-methylpiperidine methiodide)

1. Introduction

Muscarinic M_3 cholinoceptors are believed to be present in airway smooth muscle where they mediate contraction (Eglen and Whiting, 1986; Roffel et al., 1988, 1990; Hulme et al., 1990; Minette and Barnes, 1990; Eglen et al., 1991). Smooth muscle contraction mediated via muscarinic M_3 cholinoceptors is induced following the generation of inositol phosphates.

In addition to their presence on cholinergic nerve terminals, (Fryer and Maclagan, 1984; Blaber et al., 1985; Faulkner et al., 1986; Aas and Maclagan, 1990; Watson et al., 1992), small populations of the muscarinic M_2 cholinoceptor have been detected in guinea pig but not human airway smooth muscle (Mak and Barnes, 1990). However, muscarinic m_2 cholinoceptor subtype mRNA has been detected in human airway smooth muscle (Mak et al., 1992) and muscarinic M_2 cholinoceptor subtype-mediated [^{3}H]cyclic adenosine monophosphate (cAMP) formation has been demonstrated in cultured human airway smooth muscle cells (Widdop et al., 1993). Furthermore, evidence obtained from both binding and functional studies have demonstrated the existence of both muscarinic M_2 and M_3 cholinoceptor populations on guinea pig and rat airways (Fryer and El-Fakahany, 1990; Haddad et al., 1991). In addition, a more recent study has suggested that a component of muscarinic cholinoceptor-mediated guinea pig tracheal smooth muscle contraction is mediated by muscarinic M_2 cholinoceptors (Thomas and Ehlert, 1996).

The role of muscarinic cholinoceptor subtypes in mediating smooth muscle contraction in the presence and absence of epithelium-derived inhibitory factors has been of interest due to the differing effects of the epithelium on airway smooth muscle sensitivity to acetylcholine and carbachol (Eglen et al., 1991; Preuss et al., 1992). In addition, the disparate responses of tracheal tissue to acetylcholine and carbachol observed with respect to animal age (Preuss et al., 1992) further suggest differing mechanisms of action of these two agonists in the airways.

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Thus, it was of interest to determine which of the muscarinic cholinoceptor subtypes was important in eliciting smooth muscle contraction to these agonists in both epithelium-intact and epithelium-denuded tracheal tissue preparations. This study examined the effects of the muscarinic cholinoceptor antagonists atropine (non-selective), pirenzepine (M_1-selective), methoctramine (M_2-selective) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; M_3-selective) on the contractile responses of guinea pig and rat isolated tracheal smooth muscle to acetylcholine and carbachol in the presence and absence of the epithelium. The role of inositol phosphate generation in smooth muscle responses to acetylcholine and carbachol in guinea pig and rat isolated tracheal tissue was also examined.

2. Methods

2.1. Functional studies

Male guinea pigs (SR/C Tricolour) 6 weeks of age and male Wistar rats 12 weeks of age were used in this study. These ages were chosen as being the most common age at which these species were used in laboratories around the world. Animals were used in these experiments in accord with the Guidelines defined by the Australian Code of Practice for the care and use of animals for scientific purposes and the protocols approved by the Animal Ethics and Experimentation Committee of the University of Western Australia. Guinea pigs were sacrificed by cervical dislocation and exsanguinated, whereas rats were sacrificed by a blow to the head and exsanguinated. Neither method of sacrifice induced observable functional deficits in these airway preparations, with agonist potencies and maximal responses in the expected ranges. The trachea was removed and placed in ice-cold Krebs–bicarbonate buffer of the following composition (mM): NaCl 117; $NaHCO_3$ 25.0; KCl 5.36; KH_2PO_4 1.03; $MgSO_4.7H_2O$ 0.57; $CaCl_2$ $2H_2O$ 2.5 and glucose 11.1 (pH 7.4). Twelve tracheal preparations 2–3 mm in width were obtained from each animal and alternate preparations denuded of their epithelium by gentle rubbing of the luminal surface with a cotton-tipped probe. The tissue preparations were suspended under 500 mg weight tension in Krebs solution in a water-jacketed bath maintained at 37°C and gassed with 95% O_2 and 5% CO_2. At this applied force, all preparations elicited optimal, constant and reproducible contractile responses to spasmogens. Changes in isometric tension were measured by a Force displacement transducer (Grass, FTO3C) coupled to a preamplifier and recorded on a Rikadenki pen recorder (Model 1328L). Cumulative concentration–effect curves were constructed to carbachol and acetylcholine in the absence or presence of the antagonists atropine (non-selective), pirenzepine (M_1-selective), methoctramine (M_2-selective) or 4-DAMP (M_3-selective). In each animal, four preparations were used as time controls (i.e., repeated carbachol or acetylcholine curves in the absence of any antagonist) and the remaining eight were used to test responses in the presence of antagonist. For this, the first agonist cumulative concentration–effect curve was constructed in the presence of two different concentrations of antagonist, with the second curve constructed in the presence of a minimum 10-fold increase in antagonist concentration compared to the first curve. Each experiment was conducted in tissue from four different animals to account for between-animal variation.

Schild plots were constructed for each of the antagonists against both acetylcholine and carbachol in both epithelium-intact and epithelium-denuded preparations using the method of Schild (Schild, 1947, 1949). Antagonists were added to the organ bath 30 min prior to commencement of the agonist concentration–effect curves. In each experiment, acetylcholinesterase activity was inhibited by incubation of the tissues with the irreversible antagonist ecothiophate (0.1 μM) for 20 min followed by a 30 min washout and recovery period before testing.

2.2. Measurement of inositol phosphate accumulation

Total intracellular inositol phosphates were measured as previously described (Henry et al., 1992). Briefly, tracheal preparations (epithelium-intact) were incubated for 30 min at 37°C in Krebs solution gassed with 5% CO_2 in oxygen. This was then replaced with Krebs solution containing *myo*-[2-^{3}H]inositol (5 μCi) and preparations placed in a shaking water bath at 37°C for a period of 3 h. The tissues were then twice washed with oxygenated Krebs solution at 37°C for 15 min followed by a third wash for 15 min. For studies requiring the presence of antagonists, these were added during the wash phases so that the tissue was incubated in the presence of the antagonists for 30 min prior to agonist stimulation. The concentration of the antagonists used (atropine (10 nM), methoctramine (10 μM), pirenzepine (1 μM) and 4-DAMP (10 nM)) were chosen as being concentrations close to the functional pA_2 values for each of the antagonists whereas the concentration of agonists (1 mM) was chosen as it produced slightly submaximal inositol phosphate accumulation for both agonists, thereby avoiding the linear part of the concentration–effect curve. LiCl (5 mM) was added to prevent the breakdown of inositol monophosphate to inositol (Berridge et al., 1982). Tissue preparations were incubated in the absence or presence of carbachol or acetylcholine for 15 min and the reaction was then halted by the addition of chloroform:methanol (2:3), followed by the addition of equal volumes of chloroform and water to separate aqueous and lipid phases. The entire upper aqueous layer was removed and applied to an anion-exchange column of Dowex AG1-X8 resin which had been converted to the formate form in the presence of excess formic acid for 30 min and then washed three times for 10 min with distilled

water. Inositol was eluted by passing 10 ml distilled water through the column and the glycerophosphoinositol was eluted with 15 ml of solution consisting of 5 mM sodium tetraborate and 60 mM sodium formate. Finally, inositol phosphates were eluted with 10 ml of a solution consisting of 0.1 M formic acid and 0.75 M ammonium formate. This eluent was collected and dispensed into 1 ml aliquots to which 10 ml of scintillant (5.8 g/l 2,5-diphenyloxazol (PPO) in Triton X100/toluene (1:2)) was added and the radioactivity counted in a Tri-Carb liquid scintillation counter (Packard, Model 1500). Total *myo*-[2-^{3}H]inositol phosphate was calculated as dpm/mg tissue. For the construction of dose–response curves for inositol phosphate accumulation, responses are calculated as percent response of the maximal response obtained.

2.3. Data analysis

For functional experiments, the pD_2 and E_{max} values for each cumulative concentration–effect curve were estimated after curve fitting by an iterative non-linear least squares regression analysis computer program. The potency of agonists was expressed in terms of a pD_2 value (where $pD_2 = -\log_{10}(EC_{50})$ and EC_{50} is the concentration required to elicit 50% of the maximal response). Mean pD_2 values for each drug were calculated for each animal tested and final mean values were then calculated for all animals within a group to obtain a final estimate. For studies involving Schild analysis, pA_2 values for the antagonists were determined using the method of Schild (Schild, 1947, 1949). pA_2 and slope estimates were obtained following fitting of the line of best fit and the slope and intersection with the *x*-axis determined. To resolve the Schild plot of methoctramine into two components, individual Schild plots were analyzed for the lower two concentrations of antagonist and for the higher two concentrations of antagonist. Mean values calculated for each animal were then used to obtain a final estimate of the animal mean and standard error of the animal mean. The significance of differences between animal means was tested using one-way or two-way analysis of variance (ANOVA) as appropriate. Differences were considered significant when $P < 0.05$. A modified *t*-statistic was used in cases where multiple comparisons were made (Wallenstein et al., 1980).

2.4. Drugs

The drugs used in this study included: acetylcholine chloride (Sigma), carbamylcholine chloride (carbachol), atropine sulfate monohydrate (Fluka Chemie, Switzerland), 4-DAMP, methoctramine 4HCl, pirenzepine dihydrochloride (Research Biochemicals, USA), ecothiophate iodide (Ayerst Laboratories, USA) and *myo*-[2-^{3}H]inositol (Amersham, UK). For functional organ bath studies, stock solutions and dilutions of drugs were prepared in 0.9% saline and for inositol phosphate studies, they were prepared in Krebs–bicarbonate solution.

3. Results

3.1. Functional studies

3.1.1. Guinea pig

In the presence of the non-selective muscarinic cholinoceptor antagonist atropine, pirenzepine (M_1-selec-

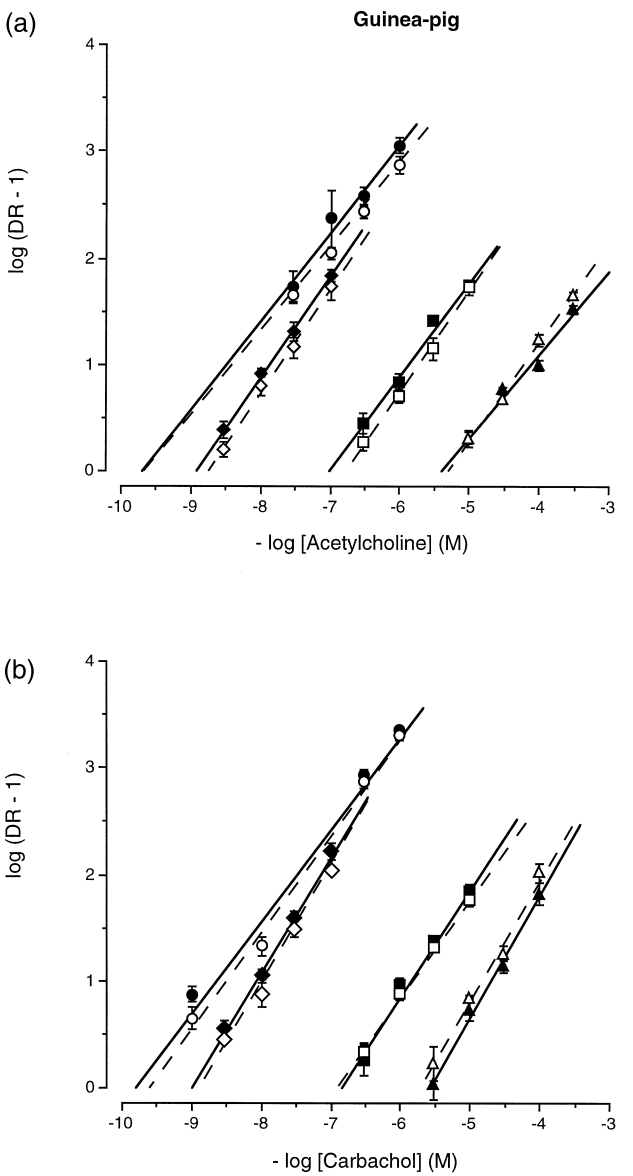

Fig. 1. Schild plots for the antagonism of (a) acetylcholine and (b) carbachol contraction in guinea pig isolated tracheal tissue by (●,○) atropine (nonselective), (■,□) pirenzepine (M_1-selective), (▲,△) methoctramine (M_2-selective) and (♦,◇) 4-DAMP (M_3-selective). Epithelium-intact tissue is represented by solid symbols and intact lines and epithelium-denuded tissue is represented by open symbols and dashed lines. Values are presented as the mean data from four animals. Vertical lines represent the standard error of the mean.

tive), methoctramine (M_2-selective) or 4-DAMP (M_3-selective), contractile responses to acetylcholine and carbachol in guinea pig isolated tracheal smooth muscle preparations were shifted to the right in a concentration-dependent manner in both epithelium-intact and epithelium-denuded preparations. The Schild plots (Fig. 1) and the derived pA_2 values (Table 1) for antagonism with atropine, pirenzepine and 4-DAMP were not significantly different between epithelium-intact and epithelium-denuded preparations. Furthermore, pA_2 values for the antagonists were similar whether acetylcholine or carbachol were used as agonist. pA_2 and slope values for methoctramine against carbachol-induced contraction were also not significantly different in epithelium-intact and epithelium-denuded preparations (Table 1). However, whilst the pA_2 values for methoctramine against acetylcholine were similar in the two tissue types, the slopes of the Schild plots were significantly different ($P < 0.05$; Student's non-paired *t*-test). In epithelium-intact tissue, the slope of the Schild plot for methoctramine against acetylcholine-induced contraction was significantly less than unity, whilst that in epithelium-denuded tissue closely approximated unity (Table 1). Resolution of the Schild plot for methoctramine against acetylcholine-induced contraction of epithelium-intact tissue using a two-site model yielded pA_2 values of 5.42 ± 0.22 ($n = 3$) and 4.87 ± 0.11 ($n = 3$) and slope estimates of 0.98 ± 0.14 ($n = 3$) and 1.14 ± 0.07 ($n = 3$) for methoctramine concentrations of 3–10 μM and 30–100 μM, respectively.

3.1.2. Rat

In the presence of atropine, pirenzepine, methoctramine and 4-DAMP, concentration–effect curves to both acetylcholine and carbachol were shifted to the right in a concentration-dependent manner in both epithelium-intact and epithelium-denuded preparations. The pA_2 values (Table 1) and the slopes of Schild plots (Fig. 2) for the antagonists were not significantly different between epithelium-intact and epithelium-denuded preparations. Schild plots for the antagonists atropine, pirenzepine and 4-DAMP all yielded slope values which approximated unity, irrespective of whether acetylcholine or carbachol was used as the agonist (Table 1). In contrast, whilst the Schild plots for methoctramine with carbachol as agonist also approximated unity and were not significantly different ($P > 0.05$) between epithelium-intact and epithelium-denuded preparations, when acetylcholine was used as agonist, these plots had slope values significantly ($P < 0.05$) less than unity (Table 1). Resolution of the Schild plot for methoctramine against acetylcholine-induced contraction in epithelium-intact tissue into a two-site model yielded pA_2 values of 6.43 ± 0.02 ($n = 4$) and 6.83 ± 0.34 ($n = 4$) and slope estimates of 1.00 ± 0.08 ($n = 4$) and 0.71 ± 0.10 ($n = 4$) for methoctramine concentrations of 3–10 μM and 30–100 μM, respectively. Although both pA_2 and slope values for methoctramine against acetylcholine-induced contraction were similar in epithelium-intact and epithelium-denuded preparations, the pA_2 values were 5- and 4.5-fold greater, respectively than that determined when carbachol was used as agonist.

3.2. Inositol phosphate accumulation

Both acetylcholine and carbachol caused concentration-dependent increases in accumulation of inositol phosphates in rat isolated tracheal smooth muscle tissue (Fig. 3). The concentration–effect curves for inositol phosphate accumulation were positioned to the right of those for smooth muscle contraction for both agonists. The potency of carbachol was greater than that for acetylcholine in stimulating both contraction and inositol phosphate accumulation, although in the latter case the differ-

Table 1
pA_2 and slope (in parentheses) values obtained for the muscarinic receptor antagonists atropine (non-selective), pirenzepine (M_1-selective), methoctramine (M_2-selective) and 4-DAMP (M_3-selective) against acetylcholine and carbachol in epithelium-intact and epithelium-denuded preparations of guinea pig and rat isolated trachea

Antagonist	Species	Acetylcholine		Carbachol	
		Epithelium-intact	Epithelium-denuded	Epithelium-intact	Epithelium-denuded
Atropine	guinea pig	9.45 ± 0.32 (0.91 ± 0.09)	$9.45 \pm 0.16^{\dagger}$ (0.86 ± 0.05)[b]	9.36 ± 0.06 (1.01 ± 0.02)	$9.22 \pm 0.11^{\dagger}$ (1.04 ± 0.03)[b]
	rat	9.67 ± 0.10 (0.94 ± 0.03)	$9.94 \pm 0.29^{\dagger}$ (0.90 ± 0.07)[b]	9.40 ± 0.11 (1.10 ± 0.02)	$9.39 \pm 0.05^{\dagger}$ (1.08 ± 0.02)[b,c]
Pirenzepine	guinea pig	6.98 ± 0.14 (0.92 ± 0.06)	$6.78 \pm 0.05^{\dagger}$ (0.95 ± 0.06)[b]	6.99 ± 0.09 (0.94 ± 0.07)	$6.92 \pm 0.08^{\dagger}$ (0.93 ± 0.03)[b]
	rat	7.13 ± 0.19 (0.93 ± 0.03)	$7.34 \pm 0.11^{\dagger}$ (0.95 ± 0.08)[b]	6.96 ± 0.13 (1.00 ± 0.12)	$6.86 \pm 0.05^{\dagger}$ (1.00 ± 0.03)[b]
4-DAMP	guinea pig	8.94 ± 0.06 (0.94 ± 0.01)[c]	$8.76 \pm 0.07^{\dagger}$ (0.97 ± 0.03)[b]	9.00 ± 0.06 (1.10 ± 0.03)[c]	$8.90 \pm 0.05^{\dagger}$ (1.06 ± 0.04)[b]
	rat	9.05 ± 0.21 (1.13 ± 0.08)	$9.20 \pm 0.10^{\dagger}$ (1.00 ± 0.05)[b]	9.08 ± 0.06 (1.11 ± 0.06)	$9.04 \pm 0.14^{\dagger}$ (1.17 ± 0.07)[b]
Methoctramine	guinea pig	5.41 ± 0.11 (0.78 ± 0.03)[c]	$5.62 \pm 0.17^{\dagger}$ (0.97 ± 0.05)[a]	5.62 ± 0.14 (1.14 ± 0.11)	$5.72 \pm 0.08^{\dagger}$ (1.14 ± 0.04)[b,c]
	rat	7.22 ± 0.31 (0.61 ± 0.06)[c]	$7.20 \pm 0.31^{\dagger}$ (0.61 ± 0.04)[b,c]	6.52 ± 0.06 (0.96 ± 0.07)	$6.55 \pm 0.12^{\dagger}$ (0.90 ± 0.06)[b]

Data is presented as mean ± S.E. of mean estimates in tissue from four animals.
[a] Indicates values which are significantly different ($P < 0.05$; Student's non-paired *t*-test).
[b] Indicates values not significantly different ($P > 0.05$) to corresponding epithelium-intact preparations.
[c] Indicates slope estimates significantly different ($P < 0.05$) from unity.

ence between concentration–effect curves for inositol phosphate accumulation did not reach statistical significance ($P > 0.05$; two-way ANOVA). The differences in potencies between the two agonists were 16- and 12-fold for contractile response and inositol phosphate accumulation, respectively. The spasmogenic potencies of carbachol and acetylcholine were 95 and 40 times greater, respectively than their potencies with respect to inositol phosphate accumulation.

The effects of atropine (10 nM), methoctramine (10 μM), pirenzepine (1 μM) and 4-DAMP (10 nM) were also examined on the inositol phosphate accumulation above basal levels in response to stimulation of tracheal tissue with carbachol (1 mM) and acetylcholine (1 mM). In guinea pig isolated tracheal smooth muscle tissue, stimulation with both agonists induced similar increases in inositol phosphate accumulation above basal. Methoctramine significantly inhibited both carbachol and acetylcholine-induced increases in inositol phosphate levels over basal. In contrast, neither atropine, pirenzepine nor 4-DAMP caused a significant decrease in inositol phosphate-induced increases above basal following stimulation with carbachol or acetylcholine, although a downward trend was observed, particularly when acetylcholine was used as the agonist (Fig. 4a).

In rat isolated tracheal tissue, the same concentration of agonist produced differing responses in inositol phosphate accumulation in isolated rat tracheal tissue, with the responses to carbachol being 2.7-fold greater than that to acetylcholine. Carbachol-induced inositol phosphate accumulation was significantly inhibited by all antagonists whereas acetylcholine-induced inositol phosphate accumulation was not significantly inhibited by any of the antagonists at the concentrations used (Fig. 4b).

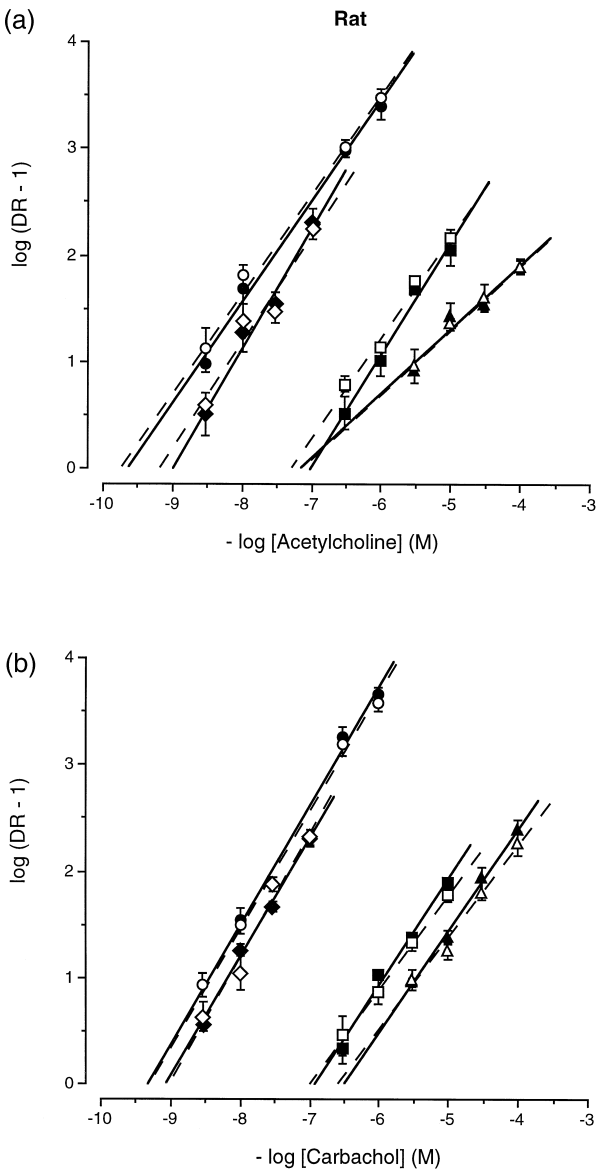

Fig. 2. Schild plots for the antagonism of (a) acetylcholine and (b) carbachol contraction in rat isolated tracheal tissue by (●,○) atropine (nonselective), (■,□) pirenzepine (M_1-selective), (▲,Δ) methoctramine (M_2-selective) and (◆,◇) 4-DAMP (M_3-selective). Epithelium-intact tissue in represented by solid symbols and intact lines and epithelium-denuded tissue is represented by open symbols and dashed lines. Values are presented as the mean data from four animals. Vertical lines represent the standard error of the mean.

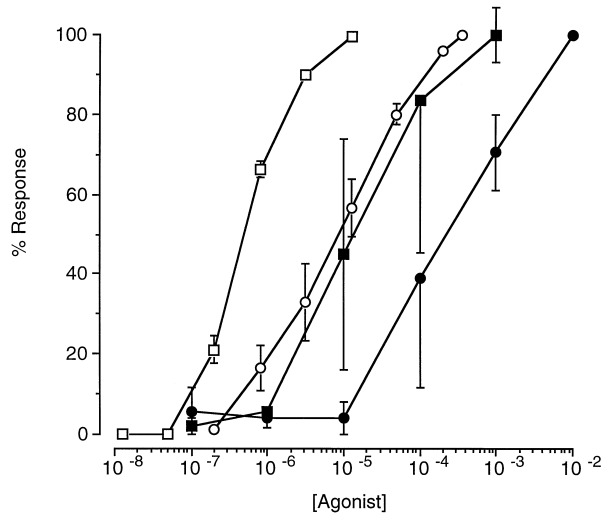

Fig. 3. Mean cumulative concentration–effect curves for (○) acetylcholine- and (□) carbachol-induced smooth muscle contraction and inositol phosphate accumulation (● and ■, respectively) in rat isolated tracheal tissue from 12-week-old animals. Values are presented as the mean data from three animals with vertical lines representing standard error of the mean.

4. Discussion

In the present study, Schild plots were constructed describing the competitive antagonism of carbachol- and acetylcholine-mediated contraction of guinea pig and rat tracheal smooth muscle by subtype-selective antagonists. pA_2 values for pirenzepine in both species were similar (6.78–7.34) and were independent of the presence of the

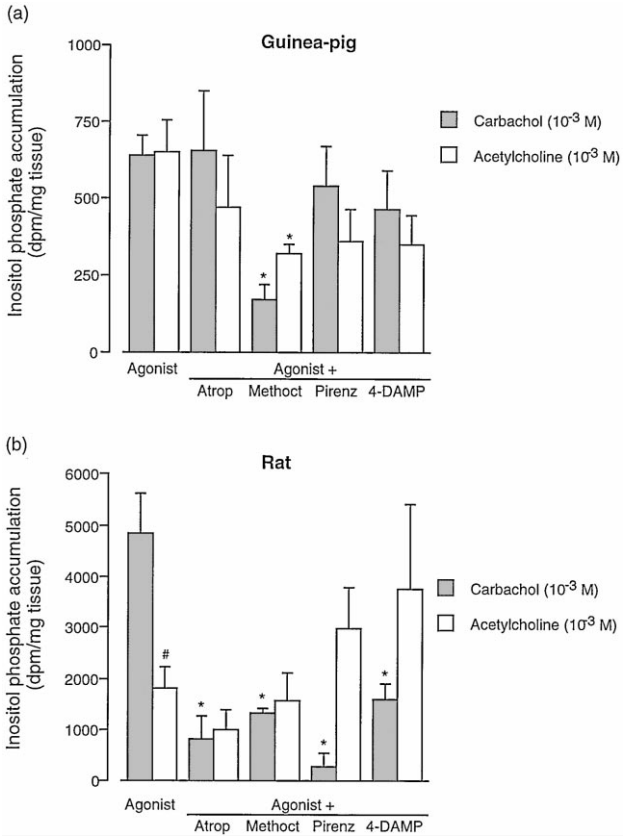

Fig. 4. Inositol phosphate accumulation following stimulation with carbachol (1 mM; filled bars) and acetylcholine (1 mM; unfilled bars) (a) guinea pig and (b) rat isolated tracheal tissue in the presence and absence of the muscarinic cholinoceptor antagonists atropine (atrop; 10 nM), methoctramine (methoct; 10 μM), pirenzepine (pirenz; 1 μM) and 4-DAMP (10 nM). # represents a significant difference between the acetylcholine and carbachol control data and * represents data significantly different from the respective control data.

epithelium, but were significantly different from previously published values for the antagonism of muscarinic M_1 cholinoceptors (approximate pA_2 of 8.1) (Hulme et al., 1990). However, these pA_2 values were consistent with antagonism of the muscarinic M_3 cholinoceptor subtypes by pirenzepine (approximate pA_2 of 6.8; Hulme et al., 1990). Published pA_2 values of approximately 9 for antagonism of muscarinic M_3 cholinoceptors by 4-DAMP (Hulme et al., 1990) compare very favourably with the values obtained in this study (i.e., 8.76–9.20) for antagonism of carbachol- and acetylcholine-induced contraction in both guinea pig and rat. Similar pA_2 values for pirenzepine and 4-DAMP to that obtained in the present study have been described in epithelium-intact (Haddad et al., 1991) and epithelium-denuded (Roffel et al., 1993) guinea pig tracheal tissue when methacholine was used as agonist. Thus, the data obtained for both 4-DAMP and pirenzepine antagonism of muscarinic cholinoceptors confirms that smooth muscle contraction in tracheal tissue in response to acetylcholine and carbachol stimulation is mediated by muscarinic M_3 cholinoceptors. The present data are in agreement with the predominantly muscarinic M_3 cholinoceptor-mediated contraction of tracheal smooth muscle (reviewed in Eglen et al., 1994).

In guinea pig trachea, the Schild plot for the M_2-subtype-selective receptor antagonist methoctramine against carbachol-induced contraction had a slope not significantly different from unity, suggesting competitive antagonism. The pA_2 values for methoctramine in epithelium-intact and epithelium-denuded preparations were 5.62 ± 0.14 and 5.72 ± 0.08, respectively which are significantly different from published values for methoctramine antagonism of muscarinic M_2 cholinoceptors of approximately 7.7–8.1 (Melchiorre et al., 1987a,b), but are similar to the values of 5.7–6.2 for the effect of methoctramine on muscarinic M_3 cholinoceptors (Melchiorre et al., 1987a,b). Similarly, in epithelium-denuded preparations, the pA_2 value for methoctramine with acetylcholine as agonist was 5.62 ± 0.17 with a slope not different from unity. In epithelium-intact preparations however, whilst the pA_2 value of methoctramine antagonism of acetylcholine-induced contraction is similar (5.41 ± 0.11), the slope of the Schild plot was significantly different from unity. Indeed, the Schild plot could be resolved using a two-site model yielding two Schild plots, both with slopes not significantly different from unity. The pA_2 values for these were 5.42 ± 0.22 and 4.87 ± 0.11 for low methoctramine concentrations (3–10 μM) and higher concentrations (30–100 μM), respectively. These values are clearly not in line with the actions of methoctramine on muscarinic M_3 cholinoceptors, particularly at the higher methoctramine concentrations, and are also significantly different from that expected when methoctramine acts on muscarinic M_1 cholinoceptors.

The reasons for this anomaly in methoctramine-induced antagonism of acetylcholine-induced contraction is unknown, but clearly the presence of the epithelium influences the activity of methoctramine at higher concentrations to reduce its antagonist potency against acetylcholine. This suggests that the contractile potency of acetylcholine is greater than would be expected in the presence of higher concentrations of methoctramine and suggests the presence of a mechanism modulating the activity of this antagonist, e.g., epithelial uptake and/or metabolism of methoctramine, or attenuation of epithelial release of a spasmolytic/inhibitory factor.

Interestingly, support for these proposals comes from a previous study which demonstrated that antagonism of muscarinic M_2 cholinoceptors caused a leftward shift in the acetylcholine concentration–effect curve in epithelium-intact but not epithelium-denuded preparations, resulting in an increase in the potency of acetylcholine and reduction in the change in acetylcholine potency observed following epithelium removal (Lev et al., 1990).

In rat isolated tracheal tissue, the pA_2 values for methoctramine against carbachol were 6.52 ± 0.06 and 6.55 ± 0.12 in epithelium-intact and epithelium-denuded

preparations, respectively and the slopes of the Schild plots were again not significantly different from unity. These pA_2 values indicate that carbachol was not mediating its actions through stimulation of muscarinic M_2 cholinoceptors, but are consistent with the actions of methoctramine on muscarinic M_3 cholinoceptors (Melchiorre et al., 1987a,b). In contrast, however, when acetylcholine was used as the agonist, pA_2 values of 7.22 ± 0.31 and 7.20 ± 0.31 were obtained in epithelium-intact and epithelium-denuded preparations, respectively, representing 5- and 4.5-fold increases in antagonist potency above those observed when carbachol was the agonist. These data appear to be consistent with the presence of muscarinic M_2 receptors. However, the slopes of these Schild plots were significantly different from unity.

Resolution of these Schild plots with a two-site model yielded Schild plots with pA_2 values of 6.43 ± 0.02 and 6.41 ± 0.03 in epithelium-intact and epithelium-denuded preparations, respectively for low concentrations of methoctramine (3 and 10 μM) with slope values not significantly different from unity. These values are significantly different from values of close to 8, which would have suggested the involvement of muscarinic M_2 receptor sites. At higher methoctramine concentrations, the slope values of the Schild plots remained significantly less than unity. As this phenomenon was observed in the absence and presence of epithelium, it is unlikely that modulation of release of an epithelial-derived relaxant or inhibitory factor (EpDIF) is responsible for the differences observed in methoctramine activity against carbachol- and acetylcholine-induced contraction. This is in line with previous observations in the rat that the airway epithelium does not play a major role in modulating responses to contractile agonists such as acetylcholine (Preuss et al., 1992). A similar study in bovine tracheal smooth muscle strips devoid of epithelium demonstrated that methoctramine produced a Schild plot with a similar pA_2 to that obtained in the present study in rats, and a slope significantly different from unity when methacholine was used as agonist (Roffel et al., 1988).

Although the pA_2 values for methoctramine are more in line with its actions on muscarinic M_1 cholinoceptors (Hulme et al., 1990), the results with pirenzepine do not support the presence of this muscarinic cholinoceptor subtype in rat isolated tracheal smooth muscle. Thus, whilst these data are generally consistent with the predominance of muscarinic M_3 cholinoceptors in rat tracheal smooth muscle, they also suggest the presence of another, as yet unidentified muscarinic cholinoceptor which perhaps has greater affinity for acetylcholine than carbachol.

From the present study, the suggestion may be made that at least one of the muscarinic cholinoceptor subtypes responsible for mediating EpDIF release from the guinea pig tracheal epithelium is of the muscarinic M_3 cholinoceptor subtype. In support of this are autoradiographic studies in guinea pig airways (Mak and Barnes, 1990) and in situ hybridization studies in human airways (Mak et al., 1992) which have demonstrated the presence of muscarinic M_3 cholinoceptors and muscarinic m_3 cholinoceptor mRNA, respectively in airway epithelium. Another study has indicated that EpDIF release in guinea pig isolated tracheal tissue is mediated via muscarinic M_3 cholinoceptors (Eglen et al., 1991). However, in that study, the authors based their antagonist studies on the inhibition of (+)-*cis*-dioxolane-induced EpDIF release, but the potency of this agonist was unchanged in guinea pig isolated tracheal tissue following epithelium removal. Secondly, the antagonist affinities described in that report for inhibition of EpDIF release are lower than the reported pA_2 values for these antagonists acting on muscarinic M_3 receptors and thus may indicate that the release of EpDIF is not solely mediated by the muscarinic M_3 receptor. It is also interesting to note that the change in acetylcholine potency observed with epithelium removal in the rabbit trachea (Lev et al., 1990) was shown to be augmented by the muscarinic M_1 receptor antagonist pirenzepine and inhibited by the muscarinic M_2 receptor antagonist gallamine, further suggesting the involvement of other muscarinic cholinoceptor subtypes.

Further evidence for the presence of heterogeneous muscarinic cholinoceptor populations in airway tissue was obtained in the studies examining *myo*-[2-^{3}H]inositol accumulation in response to acetylcholine and carbachol stimulation. The spasmogenic potencies of carbachol and acetylcholine were 95 and 40 times greater, respectively than their potencies as stimulants of inositol phosphate accumulation in 12-week-old rats. This may mean that contraction and inositol phosphate accumulation were not strongly associated or that only very small increases in inositol phosphate generation are required for contraction. A similar disparity in functional responses and inositol phosphate accumulation has previously been observed for both carbachol and the spasmogen endothelin (Henry et al., 1992). Alternatively, the data might be explained in terms of multiple muscarinic cholinoceptor subtypes linked to different second messenger systems, with inositol phosphate generation linked to muscarinic M_3 cholinoceptors playing only a minor role. To examine this proposal, we examined the effects of selective muscarinic cholinoceptor inhibition of carbachol- and acetylcholine-induced inositol phosphate accumulation in guinea pig and rat tracheal smooth muscle.

At the concentrations of antagonists used, only methoctramine inhibited carbachol or acetylcholine-induced inositol phosphate accumulation. This may have been a reflection of the antagonist concentrations used, in that although these concentrations were close to the pA_2 values obtained from the functional studies, they may not have been potent enough for the inhibition of inositol phosphate accumulation. We have already observed that the concentration–effect curves for the functional responses to carbachol and acetylcholine are shifted to the left of the inositol phos-

phate curves in the rat and thus, the antagonist concentrations used may not have had the same effects in inhibiting inositol phosphate accumulation. The fact that the non-selective antagonist atropine did not inhibit the inositol phosphate responses supports this and the fact that methoctramine was able to inhibit the responses should not be overlooked.

In contrast, carbachol-induced inositol phosphate accumulation was inhibited by all antagonists in rat isolated tracheal tissue. Conversely, acetylcholine-induced increases in inositol phosphate accumulation were not significantly altered in the presence of the antagonists. These data highlight the differences in the antagonism of carbachol- and acetylcholine-induced inositol phosphate accumulation in the isolated rat trachea.

Methoctramine had a significant inhibitory affect on carbachol-induced inositol phosphate accumulation in both guinea pig and rat tracheal tissue as well as on acetylcholine-induced responses in the guinea pig. Whilst the activity of muscarinic M_2 cholinoceptors are generally thought to act via the inhibition of adenylyl cyclase, some studies have suggested that muscarinic M_2 cholinoceptors are also coupled to inositol phosphate turnover (Ashkenazi et al., 1987; Peralta et al., 1988). However, in rat isolated tracheal tissue, methoctramine had no significant inhibitory effect on acetylcholine-induced inositol phosphate accumulation. These results give further weight to the species differences observed in both contractile responses and inositol phosphate-generating abilities of carbachol and acetylcholine seen in this study.

The present study has demonstrated that muscarinic cholinoceptor-mediated contraction of guinea pig and rat isolated tracheal smooth muscle is primarily mediated via the muscarinic M_3 cholinoceptor subtype, although the presence of another active subtype cannot be discounted. In rat isolated tracheal tissue, the presence of the epithelium does not influence the potency of the agonists or the effects of the antagonists examined. Conversely, in guinea pig isolated tracheal tissue, the presence of the epithelium significantly altered the potency of acetylcholine and the activity of the muscarinic M_2 cholinoceptor antagonist methoctramine. This suggests the possible presence of another non-M_3 muscarinic cholinoceptor population, which is sensitive to stimulation by acetylcholine, but not carbachol and which is most likely located in the epithelium. Furthermore, anomalies in the inhibitory activity of methoctramine on carbachol- and acetylcholine-mediated inositol phosphate generation suggests that these agonists may mediate their actions via different second messenger systems.

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